NON-VIRAL GENE DELIVERY:
CARRIER-MEDIATED TRANSFECTION TO RETINAL PIGMENT EPITHELIAL CELLS AND ENDOTHELIAL CELLS

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Tiivistelmä / Referat / Abstract

Gene therapy involves the delivery of exogenous DNA into the target cells in order to produce therapeutic protein or to correct a genetic defect. The use of cationic liposomes and polymers as carriers of DNA is based on observations that positively charged carriers bind to anionic DNA protecting its premature degradation and facilitating its cellular uptake in transfection. The modification of carriers and the engineering of DNA are proposed to enable efficient and prolonged protein expression after transfection.

Gene therapy is a potential treatment for age related macular degeneration (AMD). The dysfunction of retinal pigment epithelial (RPE) cells is assumed to be a significant factor in the development of AMD. The aim of this Master's thesis was to study non-viral gene delivery to RPE cells and endothelial cells using several carrier/DNA combinations. Carriers in this study were DOTAP/DOPE/PS liposomes, methacrylamide based (PDMAEMA) micelles, and anionic lipid coated DNA complexes (LCDCs). The carriers were complexed with episomal plasmid DNA or minicircles using secreted alkaline phosphatase (SEAP) gene as a marker gene. Adult retinal pigment epithelial (ARPE-19) cells, human embryonic stem cell-derived retinal pigment epithelial cells (hESC-RPE), human embryonic primary RPE cells and endothelial cells (EaHy 926) were used in transfections.

In ARPE-19 cells linear PBuA-PDMAEMA -based complexes reached the transfection efficiency of positive control whereas in human primary RPE cells star-like PBuA-PDMAEMA -based complexes were the most efficient. In human primary RPE cells, SEAP secretion lasted at least 18 days when PDMAEMA-based micelles complexed with plasmid or minicircle with cytomegalovirus (CMV) promoter were used. High nitrogen/phosphate (n/p) ratios of polyplexes decreased cell viability. DOTAP/DOPE/PS/DNA lipoplexes transfected EaHy cells with high efficiency. In hESC-RPE, lipoplexes also exceeded the transfection efficiency of the positive control and the marker protein secretion lasted ~20 days. Human elongation factor 1a (EF1a) promoter could not prevent transgene silencing. Gene delivery did not succeed with LCDCs in any transfection.

According to the results, PBuA-PDMAEMA-polymers and DOTAP/DOPE/PS-liposomes complexed with episomal plasmid or minicircles are potential gene delivery agents for further studies in AMD. More investigation is needed i.e. to confirm the transfection efficiency of the complexes in non-dividing cells.

Avainsanat / Nyckelord / Keywords

Non-viral transfection, retinal pigment epithelium (RPE), lipoplexes, polyplexes, lipid coated DNA complexes (LCDC), episomal plasmid, minicircle

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Tiivistelmä / Referat / Abstract

Geeniterapian tarkoituksena on siirtää kohdesoluihin vierasta DNA:ta tuottamaan terapeuttista proteiinia tai korjaamaan geenivirhe. Positiivisesti varautuneiden lipidi- ja polymeerikantaja-aineiden on todettu muodostavan komplekseja negatiivisesti varautuneen DNA:n kanssa suojellen DNA:ta ennenaikaiselta hajoamiselta ja parantavan DNA:n soluunottoa geeninsiirrossa. Kantajamolekyylien kehittäminen ja DNA:n muokkaaminen voivat mahdollistaa pitkäkestoinen proteiinin tuotannon kohdesoluissa.

Geeniterapia on mahdollinen tulevaisuuden hoitomuoto silmänpohjan ikärappeumaan, jossa verkkokalvon pigmenttiepiteelisolujen (RPE) toimintahäiriön oletetaan olevan yksi syy sairauden synnyssä. Tämän erikoistyön tarkoituksena oli tutkia kantaja-aineisiin perustuvia geeninsiirtomenetelmiä RPE- ja endoteelisoluihin hyödyntäen useita eri kantaja-aine-DNA-yhdistelmiä. Kantaja-aineina käytettiin DOTAP/DOPE/PS-liposomeja, metaryyliamidi (PDMAEMA) -pohjaisia misellejä ja negatiivisesti varautuneita lipidipäällysteisiä DNA-komplekseja (LCDC). Kantaja-aineet kompleksoitiin episomaalisen plasmidin tai muokatun plasmidin, pienen DNA-renkaan, kanssa. Alkaalista fosfataasia (SEAP) koodaava geeni toimi tutkimuksissa siirtogeeninä. Geeninsiirrossa käytettiin kohdesoluina verkkokalvon pigmenttiepiteelisolulinjaa (ARPE-19), ihmisen alkion kantasoluista erilaistettuja RPE-soluja, ihmisen alkion primääri-RPE-soluja ja endoteelisolulinjaa (EaHy 926).

ARPE-19-soluissa lineaarinen PBuA-PDMAEMA -kantaja-aine saavutti positiivisen kontrollin tehon geeninsiirrossa, kun taas primääri-RPE-soluissa haaroittunut PBuA-PDMAEMA osoittautui tehokkaimmiksi DNA:n kantajaksi. Primääri-RPE-soluissa SEAP-entsyymin eritys kesti ainakin 18 päivää, kun geeninsiirrossa käytettiin PDMAEMA-pohjaisia misellejä kompleksoituna episomaaliseen plasmidiin tai pieneen DNA-renkaaseen. Korkeat polymeeri/DNA suhteet osoittautuivat kuitenkin toksisiksi soluille. DOTAP/DOPE/PS/DNA-lipopleksit olivat tehokkaita geeninsiirrossa EaHy-soluihin. hESC-RPE-soluissa lipopleksit ylittivät positiivisen kontrollin tehon, ja SEAP-eritys kesti noin 20 päivää. LCDC-nanopartikkelit eivät osoittautuneet tutkimuksissa tehokkaiksi geeninsiirtäjiksi.

Tulosten perusteella PBuA-PDMAEMA- sekä DOTAP/DOPE/PS-kantajat kompleksoituina episomaaliseen plasmidiin tai pieneen DNA-renkaaseen ovat potentiaalisia geeninsiirtäjiä tutkittaessa geeniterapiaa silmänpohjan ikärappeumaan. Lisätutkimusta kuitenkin tarvitaan muun muassa varmistamaan kompleksien geeninsiirtoteho jakautumattomiin soluihin.

Avainsanat / Nyckelord / Keywords

Nonviraalinen geeninsiirto, verkkokalvon pigmenttiepiteeli (RPE), lipopleksi, polypleksi, lipidipäällysteinen DNA-kompleksi, episomaalinen plasmidi, pieni DNA-rengas

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EXPERIMENTAL WORK

ABBREVIATIONS

AAV	Adeno-associated virus
AMD	Age-related macular degeneration
ARPE-19	Spontaneously arising retinal pigment epithelia (RPE) cell line
CCV	Clathrin-coated vesicle
CHEMS	Cholesteryl hemisuccinate
CHO	Chinese hamster ovary cell line
CL22	Cationic lysine rich peptide
CMC	Critical micelle concentration
CME	Clathrin-mediated endocytosis
CMV	Cytomegalovirus
CpG	Cytosine nucleotide occurs next to guanine nucleotide
c-PGA	c-polyglutamicacid
CvME	Caveoale-mediated endocytosis
DC-cholesterol	3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DDAB	Dimethyldioctadecylammonium (bromide salt)
DGL	Dendrigraft poly-L-lysine
DNA	Deoxyribonucleic acid
DOCSPER	1,3-dioleoyloxy-2-(N(5)-carbamoyl-spermine)-propane
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOSPA	(+)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride
DOSPER	1,3-dioleoyloxy-2-(6-carboxyspermyl)propylamide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTIM	1-[2-[9-(Z)-octadecenoyloxy]]-2-[8-(Z)-heptadecenyl]-3-[hydroxyethyl]imidazolinium chloride
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
DPPE	1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine
DS	Dextran sulphate
EaHy	Human endothelial hybridoma 926 cell line
EBNA-1	Epstein–Barr virus nuclear antigen 1
EBV	Epstein–Barr virus
EF1a	Human elongation factor-1 alpha
EMA	European medicines agency
EtBr	Ethidium bromide
GAG	Glycosaminoglycan
GTPase	Guanosine triphosphate hydrolyzing enzyme
H_{II}	Two-dimensional hexagonal state

hESC-RPE	Human embryonic stem cell-derived retinal pigment epithelial cells
KALA	Amphipathic lysine-alanine-leucine-alanine peptide
Lα	Lamellar organization state
LCDC	Lipid coated DNA complex
LDL	Low-density lipoprotein
MC	Minicircle
MIDGE	Minimalistic, immunologically defined gene expression
MnSOD	Manganese superoxide dismutase
M-phase	Miotic phase of the cell cycle
N/p ratio	Nitrogen-to-phosphorus ratio
oriP	Epstein-Barr Virus replication origin
p53	Tumor suppressor p53
pBMA	Poly(butylmethacrylate)
PBuA -PDMAEMA	Poly(butyl acrylate)-poly 2-(dimethylamino)ethyl methacrylate
PCR	Polymerase chain reaction
PDGF	Pigment epithelial derived factor
PdI	Polydispersity index
PDMAEMA	Poly 2-(dimethylamino)ethyl methacrylate
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
PS	Protamine sulphate
PS-PDMAEMA	Polystyrene-poly 2-(dimethylamino)ethyl methacrylate
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
SEAP	Secreted alkaline phosphatase
S/MAR	Scaffold/matrix attachment region
S-phase	Synthesis phase of the cell cycle
SEM	Standard error mean
T_H	Hexagonal phase transition temperature
TUSC2	Tumor suppressor gene 2
VEGF	Vascular endothelial growth factor
ZP	Zetapotential

LITERATURE REVIEW

1 INTRODUCTION

Gene therapy involves the delivery of exogenous DNA into the target cells in order to produce therapeutic protein or to correct a genetic defect. Gene delivery has huge potential for treating diseases, regardless, if they are inherent or acquired: a mutated gene can be replaced and the gene therapy can take a place of conventional protein therapy (Ibraheem et al. 2014). Furthermore, the most significant attractiveness of gene therapy lies in the possibility to cure patients for their lifetime with a single intervention. Gene therapy may be considered for the treatment of diseases with following properties: the severity of condition, the lack of current treatment methods, an identified gene defect and a target organ with the sufficient access of gene delivery (Tarhini et al. 2011; von der Leyen et al. 2011; Lu et al. 2012; Alton et al. 2013; Senzer et al. 2013; Alvarez et al. 2014).

In the circulation, naked exogenous DNA is destroyed by nucleases with a half-life about ten minutes and the cell uptake of naked DNA is usually poor, thus the transfection efficiency of naked DNA is often tried to enhance by physical transfection methods (Kawabata et al. 1995; Wang et al. 2013). On the other hand, carriers of different kind have been developed to overcome the obstacle of low gene delivery efficiency. Both viral and non-viral gene delivery methods have been studied to transfer a desired gene to the target cells (Felgner et al. 1987; Wu and Wu 1987; Boussif et al. 1995; Wolfert et al. 1996; Li et al. 1998; Kay et al. 2001). Viruses are natural DNA delivery agents that can be harnessed in gene therapy (Kay et al. 2001). Retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV) have been modified for the purposes of gene delivery. However, viral gene delivery has met significant drawbacks like provoking of immunoresponses, causing carcinogenicity and having limited DNA packing capacity. Despite the encountered obstacles, Glybera, an AAV vector engineered to express lipoprotein lipase for its deficiency, was approved as the first gene delivery treatment by European Medicines Agency (EMA) in 2012 (EMA 2012).

To overcome the problems related to viral gene delivery, non-viral carrier-mediated transfection methods have been developed to mimic the transduction process of viruses. Carrier-mediated gene therapy is usually based on cationic polymers or lipids that protect DNA from degradation and enable its entry into a cell (Felgner et al. 1987; Wu and Wu 1987; Boussif et al. 1995; Wolfert et al. 1996). Hence, the success of non-viral gene therapy is largely dependent on the development of carriers that can selectively and efficiently deliver a gene to the target cells with minimal toxicity. The low transfection efficiency and possible cytotoxicity are the factors that have restricted the development of carrier-mediated transfection methods to clinical use. In addition to carrier development, DNA engineering is an area under investigation that is assumed to enable prolonged gene expression in the target cells (Darquet et al. 1997; Schakowski et al. 2001; Wong et al. 2011).

The aim of this literature review is to give an overview to the barriers related to non-viral gene delivery and to the methods to overcome these obstacles. Carrier-mediated transfection methods and DNA engineering are in the focus of this review. Finally, an insight is given to the current situation of carrier-mediated gene delivery methods in clinical trials and interesting formulations used in human studies recent years are highlighted.

2 NON-VIRAL GENE DELIVERY

In non-viral gene therapy exogenous DNA is delivered into the nucleus of a cell with carrier-mediated or physical transfection methods. Additionally, the administration of naked DNA can also be classified as a non-viral transfection method. There are many demands for a gene delivery system that need to be met to achieve efficient transfection: The target cells are reached selectively, the gene delivery system has low cytotoxicity and immunogenicity, transgene expression is sufficiently long and there is a possibility to regulate the gene expression if it is needed for the treatment of the disease (Ibraheem et al. 2014). Moreover, simple production and administration of the medicinal product are also desirable properties for a gene delivery system.

In carrier mediated or chemical gene delivery, lipids, polymers or their combinations usually act as carriers of DNA to form nanoparticles (Felgner et al. 1987; Wu and Wu 1987; Boussif et al. 1995; Guo et al. 2002). Polymer/DNA complexes and lipid/DNA complexes are called polyplexes and lipoplexes, respectively. In physical gene delivery, a physical force is used to weaken cell membranes in order to enable DNA penetration into the cells. Ultrasound, electroporation and "gene gun", based on the delivery of DNA coated gold particles by high-voltage electric charge, are examples of physical gene delivery methods (Kim et al. 1996; Yang et al. 1999; Canatella and Prausnitz 2001). Despite the interesting possibilities related to physical approaches, carrier-mediated gene delivery methods are in the focus of this literature review.

In gene delivery, many barriers are encountered before DNA can reach the target cell and its nucleus (El-Sayed and Harashima 2013). The use of cationic carriers in DNA complexation is based on the observation that cationic carrier can condense anionic DNA and thus, protect DNA and enable its entry into the cell and the nucleus (Felgner et al. 1987; Wu and Wu 1987; Boussif et al. 1995). Positively charged carrier/DNA complexes adsorb to the anionic cell membrane via electrostatic interactions which can facilitate DNA penetration into the cell and the properties of a carrier can have influence on the cell uptake route (Wong et al. 1999). On the other hand, positively charged complexes are also found to bind to proteins in the circulation and proteoglycans on the cell membrane, which can lead to complex degradation and decrease their transfection efficiency *in vivo* (Yang and Huang 1997; Zelphati et al. 1998; Ruponen et al. 2001). Therefore, negatively charged complexes are also studied in gene delivery (Guo et al. 2002; Lehtinen et al. 2011). The thorough understanding about the barriers that the gene delivery agent encounters in the body enables the development of more efficient gene delivery systems.

3 BARRIERS TO NON-VIRAL GENE DELIVERY

The first barrier, that a gene delivery system encounters, is the circulation and its components that can disintegrate the carrier/DNA complex (Yang and Huang 1997; Zelphati et al. 1998; Godbey et al. 1999). Especially albumin is the protein that has

been studied to have interactions with cationic carrier/DNA vehicles in the circulation (Zelphati et al. 1998; Dash et al. 1999). Also nucleases, the enzymes that degrade DNA, can destroy the free DNA in the circulation (Kawabata et al. 1995). Additionally, the gene delivery vehicle needs to reach the target cells, which can sometimes be challenging depending on the location of the target cells and the administration site of the gene therapy.

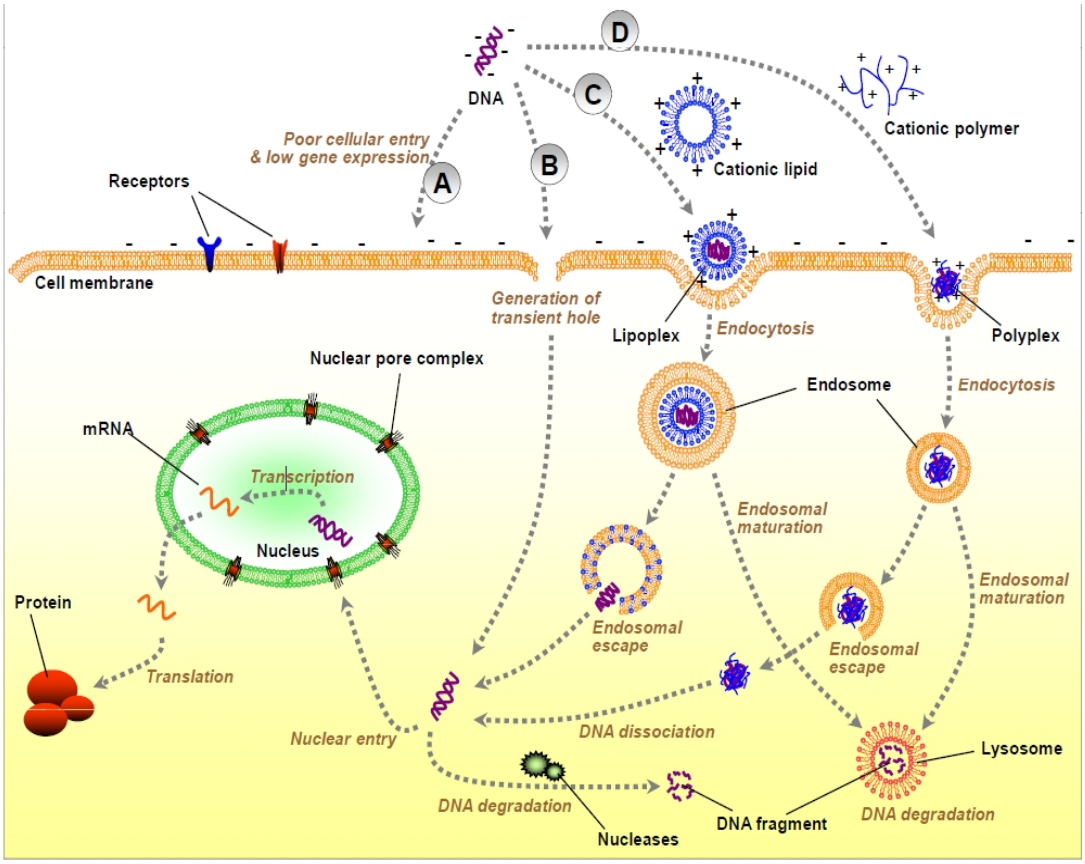

Figure 1. The cell uptake of naked DNA or carrier/DNA complexes and barriers to non-viral gene delivery. A. Naked DNA has poor ability to traverse cell membrane. B. Transfection can be facilitated with physical methods like electroporation to make transient holes in the cell membrane. C. The endocytosis process of lipoplexes. D. The endocytosis process of polyplexes. Endocytosis can be receptor mediated. Inside the cell, polyplex and lipoplex should escape from the endosome before degradation in lysosomes. After the DNA is released from the endosome, it can be degraded by nucleases in the cytoplasm. The DNA needs to enter the nucleus to be transcribed into RNA. RNA is then transferred to the cytosol and translated into the protein of interest (Wang et al. 2013).

The possible mechanisms for gene delivery vehicles to enter a cell and the barriers that naked DNA or carrier/DNA complexes encounter during a transfection process

are illustrated in Figure 1. The uptake pathways to the cell can be divided into endocytic and non-endocytic routes (Xiang et al. 2012). Clathrin-mediated endocytosis (CME), calveolae-mediated endocytosis (CvME), phagocytosis and macropinocytosis are considered endocytic pathways. Non-endocytic routes can be divided into invasive systems that cover physical methods like electroporation, and naturally occurring fusion and penetration. The size of carrier/DNA complexes can have influence on the cell uptake route and thus, on the fate of the complex in the cell (Rejman et al. 2004). Because of the many barriers that the gene delivery vehicle encounters before reaching the nucleus, it is difficult to predict which of the barriers represent the rate-limiting factor in the transfection process.

3.1 Uptake into the cell

Clathrin-mediated endocytosis (CME) is a receptor-dependent and clathrin-mediated uptake pathway into the cell (Xiang et al. 2012). For example, low-density lipoprotein (LDL) and transferrin are taken up by CME. CME process contains several phases starting from the activation of a transmembrane receptor ending to the forming of an endolysosome. The first step in CME is the binding of a ligand to its transmembrane receptor, after which intracellular clathrin-proteins polymerize and form a clathrin-coated pit on the inner side of the cell membrane (El-Sayed and Harashima 2013). This is followed by the polymerization of dynamin-2-enzyme, a kind of GTPase, which takes part in the releasing of a clathrin-coated vesicle (CCV) off the cell membrane with the diameter of ~150–200 nm.

In the cytosol, the CCV starts to lose its clathrin-coat followed by the fusion with the other vesicles to form an early endosome (El-Sayed and Harashima 2013). The pH of the early endosome begins to decrease because of the increased concentration of protons pumped by the vacuolar adenosine triphosphatase (ATPase) into the endosome. After the acidification, the endosome develops into a late endosome that fuses with a lysosome to form an endolysosome. The pH during this process is decreased from pH 6,1–6,8 of an early endosome to ~4,5 of an endolysosome. Considering gene delivery, the DNA-nanoparticle systems must escape from the endosome before the acidification process to avoid DNA degradation. So far, some non-viral carriers, like polyethylenimine (PEI) polymer and 1,2-dioleoyl-sn-glycero-

3-phosphoethanolamine (DOPE) lipid, have been found to have DNA escape assisting properties from the endosomes and their properties are described later in this review (Boussif et al. 1995; Koltover et al. 1998; Ur Rehman et al. 2013).

Caveoale-mediated endocytosis (CvME) is based on vesicles called caveoales that are located on the cell surface (El-Sayed and Harashima 2013). CvME is typical for endothelial cells, muscle cells, fibroblasts, and adipocytes. The main components of caveoales are cholesterol and caveolin-1 protein but also dynamin participates in the fission reaction of the vesicle (Henley et al. 1998; Xiang et al. 2012). After pinching off the cell membrame, caveoales fuse together and form a caveosome (El-Sayed and Harashima 2013). According to the latest studies, the intracellular pathway of caveosomes leads either to endolysosomes, Golgi apparatus, endoplasmic reticulum or transcytosis depending on the cell type (Simionescu et al. 2009; Wong et al. 2007; Xiang et al. 2012). However, an unambiguous answer to the intracellular fate of caveosomes is not yet obtained. In endothelial cells the content of caveosomes can be transcytosed, which means that molecules are transported across a cell within caveoales (Simionescu et al. 2009). If caveosomes end up in a Golgi apparatus, endoplastic reticulum or are transcytosed, it means that the cargo of the caveosome, like nanoparticles, avoid the lysosomal degradation.

Many lipoplexes and polyplexes are studied to utilize both CME and CvME pathway to enter the cell (Rejman et al. 2004; Rejman et al. 2005; Wong et al. 2007; Billiet et al. 2012). According to cell uptake studies, the uptake of several polyplexes has been shown to decrease when either CME or CvME routes are inhibited (Rejman et al. 2005; van der Aa et al. 2007). Interestingly, the transfection efficiency was decreased only when CvME was inhibited, which leads to the possibility that polyplexes are transported to the nucleus only via CvME and thus, only CvME leads to the efficient transfection. Negatively charged lipoplexes are also shown to favor CvME, while cationic lipoplexes are often taken up by CME (Billiet et al. 2012). The size of nanoparticles has also been observed to have an influence on the cell uptake route. It has been observed that the internalization of microspheres with a diameter of <200 nm occurs via clathrin-coated pits, while increasing the size over 200 nm leads to the change of the uptake route to the caveoale-mediated internalization (Rejman et al. 2004). The larger lipoplexes have been observed to have better transfection

efficiency *in vitro* compared with smaller ones, which is proposed to result from the differences in the cell uptake routes (Masotti et al. 2009).

Phagocytosis is a special type of endocytic pathway which is peculiar to professional phagocytes such as macrophages but also to the certain other cells like endothelial and epithelial cells, including retinal pigment epithelial cells (El-Sayed and Harashima 2013). The phagocytic pathway is mediated by cup-like membrane extensions to internalize large particles (>1 μm) and the shape of particles is observed to have influence on phagocytosis. Following internalization, the phagosome fuses to early endosomes and late endosomes resulting in its acidification. Especially dendritic cells and retinal pigment epithelial cells have been studied as the target cells of gene delivery in cancer and age-related macular degeneration (AMD), respectively, and phagocytosis has been suggested as a possible uptake route of nanoparticles in these cells (Mannermaa et al. 2005; Strauss 2005; Steele et al. 2011).

Macropinocytosis called also fluid-phase endocytosis is based on the nonspecific uptake of fluid-phase into the cell (Xiang et al. 2012). The destination of the macropinosomes is usually an endosome but it varies between different cell types. The studying of macropinocytosis in the cell uptake of nanoparticles is challenging because of the lack of characteristic membrane proteins or lipid coats that label macropinosomes (El-Sayed and Harashima 2013).

3.2 DNA release within cells

Endosomal escape is a significant obstacle concerning the efficiency of non-viral transfection (Varkouhi et al. 2011). While many viruses have developed efficient methods for endosomal release, non-viral transfection often suffers from deficiency to avoid lysosomal degradation (Kay et al. 2001; Varkouhi et al. 2011). Several mechanisms have been studied to enable the endosomal release of non-viral vectors like pH-buffering effect and fusion with the endosomal membrane (Boussif et al. 1995; Mönkkönen and Urtti 1998).

The proton sponge effect is typical for carriers with high buffering capacity and flexibility to swell when protonated (Varkouhi et al. 2011). Cationic polymers with nitrogen atoms, like PEI, are proposed to accept endosomal protons (Bousiff et al. 1995). The protonation is supposed to result in the inflow of chloride anions in the endosome, leading to the osmotic swelling of the endosome (Varkouhi et al. 2011). If the endosome breaks up, the complexes can be released to the cytosol.

Another example of the endosomal escape process is the flip-flop mechanism of lipoplexes, which means that contacting lipid bilayers undergo a phase change and the liposome fuse with the endosomal membrane (Zelphati and Szoka 1996; Mönkkönen and Urtti 1998). The change of long range lipid organization between Lα (lamellar organization with fluid hydrocarbon chains) and H_{II} (two-dimensional hexagonal state) states is required for lipid fusion (Figure 2) (Mönkkönen and Urtti 1998). Transition between these states takes place at hexagonal phase transition temperature (T_H). Additionally, the lipids that are able to adopt H_{II} state can also facilitate lipid fusion between two bilayers in Lα state. DOPE is an example of a lipid that has fusogenic properties (Koltover et al. 1998).

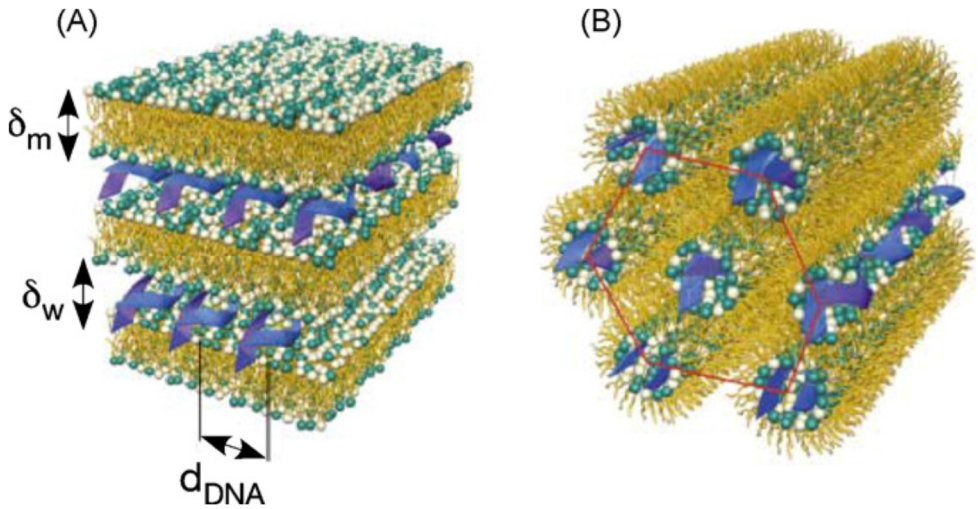

Figure 2. The schematic diagram of supramolecular structures of lipoplexes. (A) The lamellar Lα phase with alternating lipid bilayers and DNA monolayers. (B) The inverted hexagonal H_{II} phase, with cylinders consisting of DNA coated with a lipid monolayer arranged in a hexagonal lattice. (Tros de Ilarduya et al. 2010).

In addition to polymers and lipids, many kinds of cell penetrating peptides are studied to facilitate endosomal escape by forming pores in the endosomal membrane (Varkouhi et al. 2011). An interesting example is a peptide KALA (lysine-alanine-

leucine-alanine) that can efficiently cause the membrane leakage over the pH range 4,5–8, which enables the endosomal escape of DNA (Wyman et al. 1997). This phenomenon is caused by the conformational change of KALA when the pH is lowered.

3.3 Nuclear uptake of DNA and post transcriptional events

Once exogenous DNA is released into the cytoplasm, it needs to reach the nucleus to access the transcription machinery (Hsu and Uludağ 2012). The nuclear membrane regulates the transport of substances between cytosol and nucleus. The nuclear membrane contains aqueous channels called nuclear pore complexes (NPC). In the relaxed state, the pore size is ~10 nm allowing only the diffusion of small compounds like ions and metabolites through it and the transport of RNA and DNA is regulated by an energy-dependent process. Some carriers, like PEI, have been observed to promote DNA transport to the nucleus but the mechanism is not known (Bieber et al. 2002; Honoré et al. 2005).

The import into the nucleus can also be achieved during mitotic events (Hsu and Uludağ 2012). Transfection of polyplexes has shown to be cell-cycle-dependent: the nuclear entry takes place in synthesis (S) or miotic (M) phase of mitosis depending on the cell line used (Männistö et al. 2005; Grosse et al. 2006). S-phase of the cell cycle is characterized by the replication of the genome while at M-phase breakdown of a nuclear membrane temporarily removes the physical barrier of the nucleus (Hsu and Uludağ 2012). It is likely that both M- and S-phases are correlated to better nuclear uptake and more frequent transgene expression, which lead to higher transfection efficiency.

The intranuclear trafficking of DNA is the poorly understood aspect of nonviral gene delivery. Moreover, when DNA reaches the nucleus, it needs to be transcribed to RNA which is observed to be dependent at least on the location of DNA in the nucleus (Hsu and Uludağ 2012). Controversial observations have been presented on the issue whether DNA must dissociate from its carrier prior to being transcribed (Honoré et al. 2005; Hsu and Uludağ 2012).

4 CARRIERS FOR NON-VIRAL GENE DELIVERY

Polyplexes, lipoplexes and lipopolyplexes are widely investigated non-viral gene delivery systems (Felgner et al. 1987; Wu and Wu 1987; Boussif et al. 1995; Pelisek et al. 2006). Size and zetapotential measurement, the gel electrophoresis of complexes and DNA relaxation assay are examples of studies that can be conducted to predict the stability and the transfection efficiency of complexes (Bertschinger et al. 2006; Lehtinen et al. 2008).

4.1 Polyplexes

A polyplex is a complex of a cationic polymer and anionic DNA that is formed via electrostatic interactions (Tang and Szoka 1997). Polymers have shown to have several desirable properties like enhanced DNA uptake via endocytosis, protection of DNA from degradation by nucleases and improved DNA escape from endosomes (Boussif et al. 1995; Leong et al. 1998; Ur Rehman et al. 2013). However, the possible cytotoxicity of polymers needs to be taken into account when planning their use in transfections (Boussif et al. 1995; van de Wetering et al. 1998; Alhoranta et al. 2011).

The use of cationic polymers in transfections was introduced in 1987 when poly-L-lysine (PLL) with targeting moiety was shown to have transfection efficiency *in vitro* (Wu and Wu 1987). After that transfection efficiency of several natural and synthetic polymers have been studied including polyethylenimine (PEI) (Boussif et al. 1995), cationic dendrimers (Kukowska-Latallo et al. 1996), 2-dimethyl(aminoethyl) methacrylate (PDMAEMA) (van de Wetering et al. 1998) and carbohydrate-based polymers such as chitosan (Leong et al. 1998). PLL/DNA and PEI/DNA complexes can be considered as the most studied polyplexes for transfections (Tros de Ilarduya et al. 2010).

The production of polyplexes is usually simple, however, the sequence of the addition of polymers and DNA during the complexation procedure can influence on the particle size (Tros de Ilarduya et al. 2010). Cationic polymers like PEI and PDMAEMA-based polymers can condense DNA in particularly small size (150–200

nm) while some dendrimers and polylysine complexes form larger particles (>2000 nm) (Tang and Szoka 1997). The complex size can be decreased by increasing the nitrogen/phosphorus (n/p) ratio of complexes, increasing molecular weight or branching of the polymer or decreasing salt concentration of the medium (Tang and Szoka 1997; van de Wetering et al. 1998; Honoré et al. 2005). For efficient condensation of DNA high enough n/p ratio needs to be used to form stable complexes but similarly the cytotoxicity of the complexes with high n/p ratios needs to be considered (van de Wetering et al. 1998; Bertschinger et al. 2006; Alhoranta et al. 2011).

Poly-L-lysine (PLL) has been studied as a transfection carrier for decades because of its excellent DNA condensing properties (Wu and Wu 1987; Kodama et al. 2014). PLL is linear polypeptide composed of amino acid lysine which makes it biodegradable (Tros de Ilarduya et al. 2010). PLL is observed to bind to plasma proteins, especially to albumin, which leads to its fast clearance from the blood circulation *in vivo* (Dash et al. 1999). Additionally, its transfection activity alone is poor and therefore PLL is usually modified to improve its efficiency in transfections: PLL has been coated with polyethyleinglycol (PEG), targeting ligands have been added on it, it has been combined with fusogenic agents or histidine groups have been introduced to PLL to have proton sponge effect (Wu and Wu 1987; Wolfert et al. 1996; Lee et al. 2002; Hwang et al. 2014).

An example of the recent approaches in gene delivery with PLL is dendrigraft PLL (DGL) polymers which are, as opposite to many other dendrimers, biodegradable (Cottet et al. 2007; Kodama et al. 2014). Despite promising results *in vitro* and *in vivo* transfection studies, DGL polymers have been found to induce toxicities, which has led to an experiment to coat them with anionic polymer c-polyglutamicacid (c-PGA) to neutralize the excess of positive charges (Kodama et al. 2014). DGL coated with PGA has found to have better transfection efficiency compared with PLL or DGL alone *in vitro*. However, more investigation is needed to figure out if they have potential for clinical applications.

Many kinds of modifications of PEG-PLL polymers have been done starting from Wolfert and co-worker's successful studies (Wolfert et al. 1996). One example is the

production of the block copolymers of polyethylene glycol and poly-L-lysine linked by a pH-responsive poly-L-histidine group (PEG-$CH_{12}K_{18}$) (Boylan et al. 2012). This PEG-$CH_{12}K_{18}$ has proved to have potential as a carrier probably because of the cover of PEG in the circulation and the ability of poly-L-histidine to accept endosomal protons and thus, enable endosomal escape.

Polyethylenimine (PEI) is a cationic polymer which can be found as linear and branched macromolecule and the backbone of PEI is composed of two carbons followed by one nitrogen atom which can be protonated (Figure 3). This leads to the effective buffering capacity of PEI at physiological pH (Boussif et al. 1995). However, pK_a value of PEI is not determined because of several, even over 1000, amine groups of PEI depending on its molecular weight.

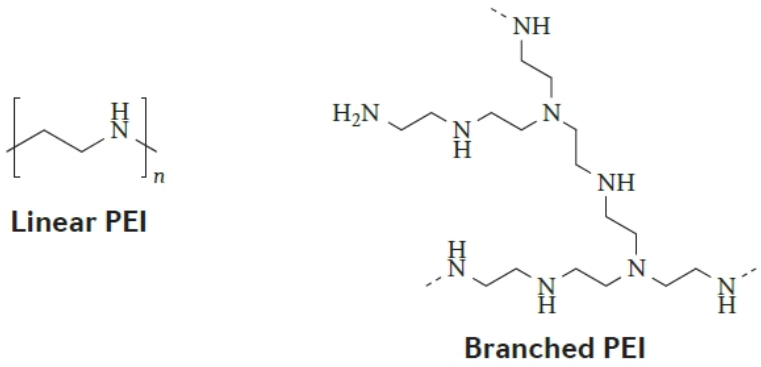

Figure 3. The chemical structures of linear and branched PEI (Yin et al. 2014).

PEI can form stable complexes with anionic DNA although branched PEI 25 kDa condenses DNA more efficiently compared with linear PEI 22 kDa (Dunlap et al. 1997; Bertschinger et al. 2006). Serum has been observed to bind and inactivate polyplexes, however, the use of a more concentrated PEI/DNA solution, a different PEI/DNA ratio or a modified surface of the complexes may counteract the negative effects of serum on transfection efficiency (Godbey et al. 1999; Moret et al. 2001). Free PEI is harmful to the cells probably because of its ability to permeabilize membranes but its toxicity is decreased when it is complexed with DNA (Boussif et al. 1995).

PEI/DNA complexes can enter the cell via a CME and a CvME route (Rejman et al. 2005). In the earlier studies, the proton sponge property of PEI has been assumed to protect DNA from degradation in lysosomes (Boussif et al. 1995). It was hypothesized that PEI accepts protons, which has impact on the pH of endolysosomes. This was proposed to lead to the inactivation of degradative enzymes, after which endolysosomal bursting occurs and PEI/DNA complexes are released. However, the hypothesis of endolysosomal bursting is questioned in the recent studies. Ur Rehman et al. (2013) have studied the delivery of polyplexes into the cell by spinning disk confocal microscopy. They observed that PEI/DNA polyplexes did not cause endosomal lysis but a local destabilization of the endosomal membrane. That was possibly induced by osmotic or mechanical effect at the site of polyplex-endosomal membrane interaction. On the other hand, Rejman et al. (2005) concluded that PEI/DNA complexes can reach the nucleus only via CvME route which does not necessarily lead to the lysosomes. The cell entry mechanism can vary between the particles with varying size and in different cell types, which can lead to different results in cell uptake studies. Despite the cell uptake route, DNA has been observed to enter the nucleus in the PEI/plasmid complex, which means that DNA does not necessarily need to separate from the PEI before nucleus entrance (Honoré et al. 2005).

Methacrylamide-based polymer poly 2-(dimethylamino)ethyl methacrylate (PDMAEMA) is a pH sensitive compound used as a carrier in transfection studies. PDMAEMA is a water-soluble vinyl polymer with tertiary amine side groups formed by radical polymerization (Figure 4A) (van de Wetering et al. 1998). The pK_a value of the side groups is about 8, which leads to their protonation at physiological pH. Cationic PDMAEMA condenses DNA and the transfection efficiency of the complexes is observed to be dependent on the PDMAEMA/DNA ratio, the molecular weight of PDMAEMA and the PDMAEMA-copolymer modifications. The high molecular weight of the polymer (>60 kDa) has shown to improve the transfection efficiency. The significant benefit compared with many other polymers and liposomes for transfection is that PDMAEMA-based polyplexes are resistant to the disintegrative impact of serum (van de Wetering et al. 1998).

Free PDMAEMA has negative impact on the cell viability, which is observed to decrease the transfection efficiency when the high n/p ratios of PDMAEMA/DNA complexes are used (van de Wetering et al. 1998). Because of the high toxicity of PDMAEMA, the formation of PDMAEMA-based block copolymers has been an interesting area of investigation to produce safer PDMAEMA-based carriers (Funhoff et al. 2005; Alhoranta et al. 2011). A block copolymer is a polymer made of different repeating chemical monomers attached to each other by various types of covalent bonds (Hamidi et al. 2012). Block copolymers have ability to self-assemble and form micelles in selective solvents, which can be utilized in production of carrier/DNA complexes. Copolymers can be divided into star-like, linear, grafted and cross-linked copolymers, which have different properties like critical micelle concentration (CMC) and hydrodynamic dimensions.

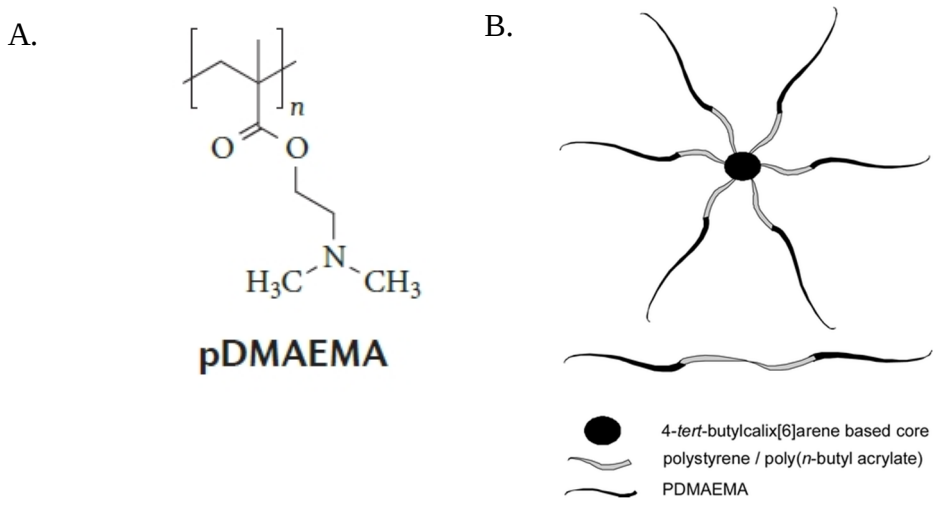

Figure 4. A. The chemical structure of PDMAEMA (Yin et al. 2014). B. The structure of linear and star-like PBuA-PDMAEMA and PS-PDMAEMA copolymers. Linear copolymers are formed from three blocks and star-like copolymers have six arms (Alhoranta et al. 2011).

Funhoff et al. (2005) have produced triblock copolymers with a PDMAEMA-block, a hydrophopic poly(butylmethacrylate) (pBMA) part and a PEG block. The purpose of PEG is to protect the cells from the cytotoxic properties of PDMAEMA. The complexes showed lower cytotoxicity but they could not reach the transfection efficiency of non-pegylated PDMAEMA. The pegylation of the polymer or neutral zetapotential of complexes might be the reasons for the lower cell uptake. Alhoranta

et al. (2011) have produced linear and star-like structured PDMAEMA block copolymers. Calix[6]arene was used as a core molecule to form star block copolymers with six arms. A hydrophobic core was formed from either polystyrene (PS) or poly(*n*-butylacrylate) (PBuA) and outer block consisted of cationic PDMAEMA (Figure 4B). Linear block copolymers are formed from three blocks having polystyrene or poly(*n*-butylacrylate) in the middle and PDMAEMA blocks on the edges. In transfection studies, the change in architecture of copolymer was observed to have an influence on the transfection efficiency and linear PBuA-PDMAEMA -based polyplexes were the most effective and promoted high cell viability.

4.2 Lipoplexes

Lipoplexes are produced by the interaction of cationic liposomes with DNA. Cationic liposomes have many desirable properties concerning transfection like biodegrability, low toxicity and non-immunogenicity (Chesnoy and Huang 2000). However, the positive results are often obtained only *in vitro* experiments because of the sensitivity of lipoplexes to serum components (Yang and Huang 1997; Zelphati et al. 1998). The synthesis of cationic lipid DOTMA and the promising results of the transfection studies *in vitro* in 1987 cleared the way for the production of many cationic lipids for gene delivery (Felgner et al. 1987). Lipoplexes are widely studied *in vitro* and many applications are taken as far as to clinical trials (Felgner et al. 1987; Ruponen et al. 1999; Masotti et al. 2009; Tarhini et al. 2011; von der Leyen et al. 2011; Lu et al. 2012; Senzer et al. 2013).

Liposomes are formed spontaneously from phospholipids when the lipids self-assemble to vesicles in the presence of aqueous medium (Chesnoy and Huang 2000). Conventional methods for the production of liposomes are thin film hydration, detergent depletion, ether/ethanol injection, reverse phase evaporation and emulsion methods. Liposomes are usually produced from cationic lipids that can be combined with neutral helper lipid (Ruponen et al. 1999; Masotti et al. 2009 Pozzi et al. 2014). Cationic lipids are composed of a hydrophobic anchor, a linker and a head group (Figure 5). Oleyl chain (C18:1) is the most common unsaturated alcyl chain and it is part of well-known lipids like DOTAP and DOTMA. Cholesterol derivatives are

often used in the mixture of double-chain lipids to stabilize liposomes (Masotti et al. 2009; Pozzi et al. 2014).

The linker of a lipid is located between a hydrophobic anchor and a head group (Chesnoy and Huang 2000). Three carbon glycerol skeleton is used as a linker in double-chain hydrocarbons. Biodegradable ester, amide or carbamaoyl bonds or non-biodegradable ether bonds can be used in linkers. The head group of cationic lipids have one or more positive charges. Monovalent lipids have a tertiary or a quaternary ammonium group that can be chemically modified.

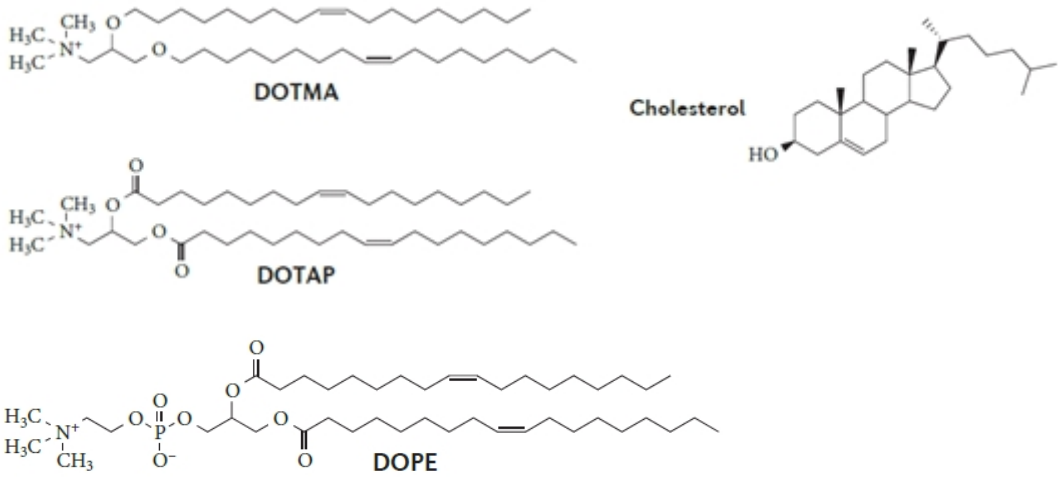

Figure 5. The chemical structure of cationic lipids DOTMA and DOTAP and neutral lipids cholesterol and DOPE (Yin et al. 2014).

First synthetized cationic lipid DOTMA is still used in transfection studies and it is a part of commercial product Lipofectin which is composed of DOTMA and neutral lipid DOPE (Masotti et al. 2009). Other examples of cationic lipids are DOTAP and DDAB (Chesnoy and Huang 2000). DC-cholesterol is cholesterol-based cationic lipid which is often studied in transfections combined with other cationic lipids. Cationic DOSPA is combined with DOPE in commercial product Lipofectamine. In cationic lipoplexes, DNA can be packed into the core of the liposome or between the lipid layers in multilamellar structure where it is in safe from the degradation (Figure 2). The incubation time and lipid concentration have been observed to have impact into lipoplex structure.

The widely used helper lipid in lipolexes is DOPE that is a neutral molecule having property to form a hexagonal phase of the lipid layer (Chesnoy and Huang 2000). Formation of the hexagonal phase in lipoplexes following interaction with the anionic lipids of endsosomes is crucial for DNA release to the cytoplasm. DOPE has been shown to control the spontaneous curvature of the lipid monolayer, which leads to its fusogenic property (Koltover et al. 1998). Koltover et al. (1998) have observed that in DOTAP/DOPE lipoplexes, the natural curvature of the lipid monolayer is driven negative because of differences in lipid shapes: cationic lipid DOTAP is cylindrically shaped whereas DOPE is conelike. This induces the transition from lamellar to hexagonal geometry. It is assumed that the negative curvature of lipoplex inside the anionic endosome leads to the expelling of DNA outside of the endosome, which saves DNA from lysosomal degradation. When compared DOPE with DOPC lipid, that cannot form the hexagonal phase, DOPE improved the transfection efficiency of lipoplexes (Zhou and Huang 1994). Therefore, it can be concluded that it is dependent on the geometry of the lipids used in the liposome if the hexagonal phase is achieved.

The influence of a DNA condensing agent protamine sulfate (PS) combined with liposomes has been studied on the transfection efficiency (Sorgi et al. 1997; Li et al. 1998; Mannermaa et al. 2005). PS is sulphated, arginine-rich peptide (M_w≈4000–4250) that is usually purified from the mature testes of fish (Sorgi et al. 1997). In sperm, protamine binds DNA to form compact structure and helps to deliver DNA to the nucleus of the egg. Thus, it has been considered as an interesting compound in gene delivery. In addition to its many desirable properties, it has been used for decades as a heparin antagonist and an excipient in insulin formulations, where it has shown its low toxicity and immunogenicity.

The influence of PS on the transfection efficiency is studied with liposomes formed from DC-cholesterol, Lipofectin, Lipofectamine, DOTAP or DOTAP/DOPE (Sorgi et al. 1997; Li et al. 1998; Mannermaa et al. 2005). PS has shown to improve transfection efficiency compared with DNA-condensing agent PLL complexed with DS-cholesterol liposomes (Sorgi et al. 1997). Additionally, liposomes complexed with PS/DNA have showed to have higher transfection efficiency compared with

liposomes alone when DS-cholesterol, Lipofectin, and DOTAP/DOPE liposomes have been studied.

4.3 Lipopolyplexes

In recent years, studies have been published about the combinations of cationic polymers and liposomes to form complexes called lipopolyplexes (Pelisek et al. 2006; Anwer et al. 2010; Ewe et al. 2014). Lipopolyplexes are forumulated to have a polymer/DNA core with a lipid shield that can be PEG-modified to give more protection to the complex (Pelisek et al. 2006; Lehtinen et al. 2008; Ewe et al. 2014). The lipid shield can be either cationic, anionic or neutral depending on the used lipids. It is notable that despite the combination of both polymers and lipids, the particle size of lipopolyplexes usually stays between 100–250 nm depending on the composition of the lipid shield. In addition, lipopolyplexes have been shown to avoid the aggregation of complexes during the storage which is often observed as a problem especially with polyplexes.

The cationic lipopolyplexes have shown to improve transfection efficiency compared with corresponding lipoplexes and polyplexes (Pelisek et al. 2006). The lipopolyplex formulation with a PEI/DNA core shielded with cationic multivalent DOCSPER or DOSPER lipid has been shown to have even 400-fold higher transfection efficiency compared with the corresponding lipoplexes or polyplexes. The reasons for promising results are assumed to lie in efficient endosomal escape when lipids and polymers are combined. However, the mixing order of the components of lipopolyplexes has been shown to affect significantly the transfection efficiency and the DNA condensation with a polymer needs to precede the lipid coating to achieve high transfection efficiency (Pelisek et al. 2006).

Anionic glycosaminoglycans (GAGs) are supposed to have interactions with positively charged polyplexes and lipoplexes, which has impact on the cellular entry and stability of DNA complexes (Ruponen et al. 2001). GAGs are long, negatively charged polysaccharides with sulphate groups (Tomar 2008). Because of their negative charge, they can trap positively charged ions and water molecules to form a gel around the cell that is a support of the extracellular matrix.

Anionic lipid-coated DNA complexes are studied to avoid the undesirable interactions with carrier/DNA complexes and GAGs (Guo et al. 2002; Lehtinen et al. 2008). They are developed to cover a positively charged polymer/DNA core from the interactions with GAGs by coating the polyplex with an anionic lipid shield. CHEMS lipid is negatively charged in high pH value accepting protons when pH decreases. Thus, the DOPE/CHEMS lipid shield is supposed to become fusogenic in the acidic environment of endosomes. Despite the interesting theory, contradictory results have been obtained from the transfection studies (Guo et al. 2002; Lehtinen et al. 2008). Guo et al. (2002) obtained positive results when they compared the transfection efficiency of lipopolyplexes with corresponding polyplexes without shielding. On the other hand, the transfection efficiency of anionic complexes was not as high as non-coated PEI-DNA complexes according to the studies of Lehtinen et al. (2008). The difference in cell lines used in transfections might had impact on varying results.

5 DNA ENGINEERING

Gene delivery has been suggested as an optimal therapy for diseases that need continuous medication. That leads to the requirement for long term gene expression in target cells. However, the integration of a transgene to the chromosomes of a target cell is not usually reached with non-viral transfection methods. On the other hand, if the integration is random, it can be a mutagenic event and thus undesirable (Kay et al. 2001). The silencing of non-integrated genes is often observed as a problem with exogenous DNA (Liu et al. 2009). The engineering of DNA is proposed to enable prolonged protein expression after transfection. In Table 1 a summary of DNA forms and modifications used in non-viral transfections is provided.

Table 1. DNA forms and modifications used in non-viral transfections.

	Benefits	**Drawbacks**
Plasmid 1)	- simple and well-known production - alternative promoters - possible to modify to have episomal replication (EBNA-1)	- bacterial sequences
S/MARs 2)	- destabilize DNA duplex structure → expression is increased - replication in host cells	- opposite results in transfection efficiency studies
Minicircle 3)	- small size (~2–4 kbp) → higher diffusion coefficient → effective endocytosis → better and longer transfection efficiency - no immunostimulatory sequences	- time-consuming, labor intensive production
Linear DNA 4)	- small size (~1–1,2 kbp) - narrow conformation - no immunostimulatory sequences - production possible synthetically by PCR → endotoxin free	- random integration to the genome possible

Abbreviations: Epstein-Barr virus nuclear antigen-1 (EBNA-1); polymerase chain reaction (PCR); kilobasepairs (kbp)

1) Wendelburg and Vos 1998; Clark 2005; Qin et al. 2010
2) Bode et al. 2006; Wong et al. 2011; Klausner et al. 2012
3) Darquet et al. 1997; Kay et al. 2010
4) Schakowski et al. 2001; Hirata et al. 2007

5.1 Plasmids

Plasmids are circular, double-stranded molecules of DNA which can replicate autonomously in their host cells (Clark 2005). They have been found both prokaryotic and eukaryotic cells but most plasmids inhabit in bacteria. Plasmids are negatively charged because of the anionic phosphate groups of DNA. They can have the topology of supercoiled, open-circular or linear, but in transfection studies supercoiled plasmid has observed to have the highest transfection efficiency in mammalian cells (Cherng et al. 1999). That is probably caused by the lower susceptibility of supercoiled DNA to nucleases. In addition to topology, the size of plasmid has observed to have an influence on the transfection efficiency: smaller plasmid molecules have shown to have higher efficacy in transfection compared with larger ones (Kreiss et al. 1999). Nuclear entry via the nuclear pores might be dependent on plasmid size, which has an influence on the transfection efficiency especially in non-dividing cells.

Usually viral cytomegalovirus (CMV) promoter is used in non-viral transfection since it has been shown to have high expression level of transgenes compared with many other viral promoters (Li et al. 1992; Qin et al. 2010). Because of the viral background of CMV promoter, the duration of the protein expression regulated by human origin promoters is considered longer in transfections to human stem cells because of the silencing of genes with CMV promoter (Liu et al. 2009). The other examples of promoters used in mammalian cells are the simian virus 40 early promoter (SV40), human ubiquitin C promoter (UBC), human elongation factor 1a promoter (EF1a), mouse phosphoglycerate kinase 1 promoter (PGK) and chicken b-Actin promoter coupled with CMV early enhancer (CAGG) (Li et al. 1992; Qin et al. 2010).

One approach to modify plasmid to improve the transgene expression duration is to modulate the origin of plasmid replication (ori) region. Epstein–Barrin virus (EBV) -based vectors carry two essential elements for the replication of the EBV genome: oriP and Epstein-Barr virus nuclear antigen-1 (EBNA-1) gene coding a replication initiation factor (Lindner and Sugden 2007). EBV has ability to maintain its genome as an extrachromosomal replicon in the cell and to steal the cellular DNA replication machinery for its replication using oriP and EBNA-1. oriP is a region of (EBV) chromosome that supports the episomal replication and stable maintenance of plasmids in human cells when EBNA-1 protein is bound to it. This property can be used for plasmid engineering combining the oriP region and the gene coding EBNA-1 in plasmid used in transfection (Wendelburg and Vos 1998). Plasmids expressing EBNA-1 can episomally replicate in the host cells during a cell division, which enable the cell to maintain the plasmid number.

Scaffold matrix attachment regions (S/MARs) are AT-rich sequences that are the sites of attachment between the nuclear matrix and DNA in eukaryotic cells (Bode et al. 2006). The nuclear matrix is formed by a network of proteins that consists of nuclear pores, a nuclear lamina, a residual nucleolus and an internal fibrillar–globular network. Attaching to the nuclear matrix, S/MARs are suggested having a role in chromatin organization, replication and gene expression (Käs et al. 1993). They can prolong gene expression by destabilizing DNA duplex structure which is caused by base-unpairing regions (Bode et al. 2006). Furthermore, S/MARs are also observed

to drive plasmid DNA replication. Incorporation of S/MARs into a plasmid has enabled plasmid to replicate and establish mitotic stability in hepatocytes at a detectable level for three months in adult mice (Wong et al. 2011). Contrary, Klausner et al. (2012) have studied S/MAR plasmid for corneal transfection in rats. They compared the transfection efficiency of nanoparticles with different plasmid types and noticed that transfections with S/MAR plasmid did not achieve higher transfection efficiency compared with plasmids without S/MAR sequences.

5.2 Minicircles

The modifications to polyadenylation, a transcriptional termination sequence and the backbone elements of plasmids have been shown to increase the non-viral transfection efficiency *in vivo* (Hartikka et al. 1996). In addition, the small size of DNA molecule has been observed to have benefits in transfection (Kreiss et al. 1999). These factors have led to plasmid engineering to produce recombinant supercoiled DNA molecules, called minicircles. The minicircles lack of bacterial origin backbone sequences, like antibiotic resistance gene and immunogenic unmethylated CpG motifs, containing only the therapeutic expression cassette (Darquet et al. 1997).

The production of minicircles is based on a parental plasmid which contains a cloned transgene expressing cassette in the bacterial backbone (Figure 6) (Kay et al. 2010). This parental plasmid has two attachment (attB and attP) sites of ΦC31 integrase that are in the same orientation in the same replicon. According to the protocol of Kay et al. (2010), parenteral plasmids are introduced to a genetically modified *E. coli* strain where the replication takes place. The replication is performed by ΦC31 integrase that converts a parenteral plasmid at the temperature of 37 °C to a minicircle with a transgene and a miniplasmid with bacterial backbone. After the replication, an I-SceI endonuclease degrades undesirable miniplasmid when the endonuclease targets the special degradation sites presented only in the miniplasmid and parenteral plasmid. Both ΦC31 integrase and I-SceI endonuclease are under the control of the presence of arabinose, thus the reactions can be regulated.

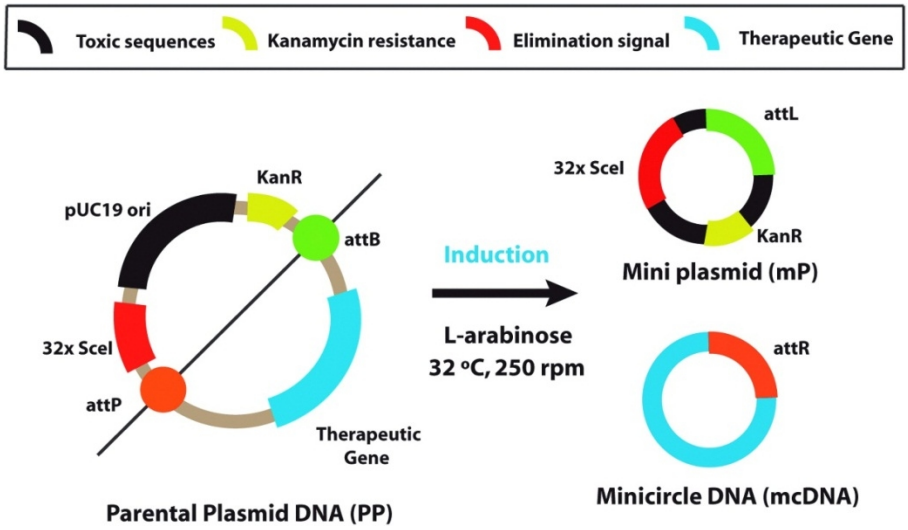

Figure 6. The figure shows the production model of minicircles. Two attachment (attB and attP) sites of ΦC31 integrase are in the same orientation in the same replicon. The replication is performed by ΦC31 integrase when L-arabinose is present. Parenteral plasmid and mini plasmid are degraded by endonuclease after minicirlce formation (Gaspar et al. 2014).

Darquet et al. (1997) have studied the transfection efficiency of minicircles compared with plasmid in several mammalian cell lines. They found that the production of a marker protein was from two- to ten-fold higher compared the transfection efficiency of minicircle with plasmid. Chen et al. (2003) have shown that even 560-fold production of marker protein can be achieved with the transfection of minicircle compared with plasmid in mice. The transgene expression was observed for three weeks.

In addition to transfection efficiency issues, minicircles are considered safer compared with the plasmid DNA (Darquet et al. 1997). The reason assumed to lie in the shortage of CpG bacterial sequences that act as the adjuvants of the immune system. The other significant factor is the risk of recombination of an antibiotic resistance gene with plasmids in endogenous *Enterobacteriacae*, which could lead to a selective advantage of these bacteria if the antibiotic in question is administered.

5.3 Linear DNA

Linear DNA vectors are developed to improve the safety and the efficiency of transfections and they can be divided into polymerase chain reaction (PCR)

amplified DNA and minimalistic, immunologically defined gene expression (MIDGE) vectors (Schakowski et al. 2001; Hirata et al. 2007). Synthetic, PCR amplified DNA and MIDGE vectors contain a promoter, a coding gene and a RNA stabilizing sequence (Figure 7). As opposite to PCR products, the units of MIDGE are combined with two short oligonucleotide hairpin structures that protect it from the degradation by nucleases (Schakowski et al. 2001). MIDGE vectors are produced from plasmid DNA digesting the plasmid with *Eco*RI endonuclease enzyme and ligating the formed fragments to hairpin oligoDNAs. OligoDNAs are produced by chemical DNA synthesis. Unligated fragments are digested and MIDGE vectors are purified by anionic-exchange chromatography.

Figure 7. MIDGE vector is composed of a promoter, a transgene, a RNA stabilizing sequence (SV40 PA) and hairpin-like oligoDNAs (Schakowski et al. 2001).

The transfection efficiency of MIDGE vectors is compared with plasmid *in vitro* studies with encouraging results (Schakowski et al. 2001). Higher transfection efficiency of MIDGE lipoplexes was observed in comparison with plasmid lipoplexes. The low content of CpG sequences is suggested improving transfection efficiency. In addition, MIDGE vectors complexed with liposomes have been studied in DNA vaccination in mice (Endmann et al. 2014). Endmann et al. (2014) observed high immune responses which were explained by the high expression of transgene coding immunostimulatory cytokine. The vector DNA was detected still after two months after transfection in several tissues. However, more studies about the possible integration of MIDGE to the host cell genome are needed.

6 CARRIER MEDIATED GENE DELIVERY IN CLINICAL TRIALS

None of the medicinal product using non-viral transfection methods has yet a marketing authorization in European Union although interesting clinical trials have been conducted in the past few years (Table 2). Concerning both viral and non-viral

transfection methods, it has been estimated that globally over 2000 gene therapy clinical trials have been approved from January 1989 till June 2014 (Journal of Gene Medicine 2014). The first clinical trial using gene delivery was conducted in 1989 and it was a gene-marking study of tumor-infiltrating lymphocytes by retroviral transdution (Rosenberg et al. 1990). During the recent years, 100±20 gene therapy clinical trials have been conducted annually (Journal of Gene Medicine 2014). However, AAV vector based Glybera is the only gene therapy medicinal product with the marketing authorization in European Union so far (EMA 2012).

Because the data of all the approved clinical trials is not publicly available, it is impossible to gather the comprehensive summary of approved or conducted clinical trials. However, in Figure 8 collects the information on the approved gene therapy clinical trials worldwide classified by the carriers or the indications but the figures are only approximates. Viral transduction can be considered the most investigated gene delivery method in clinical trials during the time (Figure 8). The studying of non-viral transfection methods is still in the beginning and the administration of naked DNA is the most used non-viral transfection method (17,7 %), while lipofection covers only 5,3 % of gene therapy clinical trials. The common indications in gene therapy clinical trials are cancer, infectious and cardiovascular diseases and monogenic diseases (Figure 8).

Clinical trials can be divided in four phases (I–IV) although the border between the phases is not always clear (EMA 1998). In phase I trials, researchers usually test a new drug in a small group of healthy volunteers or patients for to evaluate its safety, tolerability, pharmacokinetics and possibly pharmacodynamics. The aim of phase II studies is usually to explore the therapeutic efficacy of the study drug in patients and to determine the dose for phase III trials. In phase III trials, the drug is given to large groups of patients to confirm its effectiveness and safety and provide a basis for marketing approval. After the drug has been approved, phase IV studies are done to gather information on the drug's effect in various populations after long-term use. Phase IV trials are not considered necessary for approval of the drug but they can be used for optimizing the drug's use.

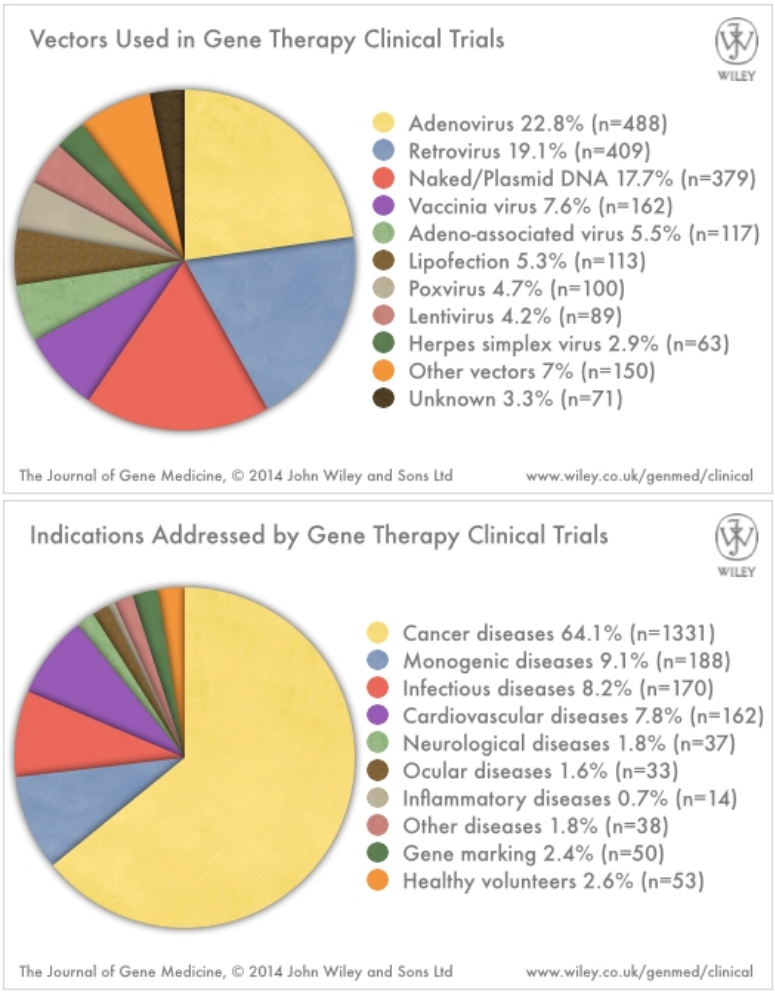

Figure 8. The approximate division between gene delivery carriers and indications in approved clinical trials worldwide between Jan 1989–Jun 2014 (Journal of Gene Medicine 2014).

In Table 2 is collected clinical trials in which carrier-mediated gene delivery has been studied and the results have been published in the past five years. All the clinical trials in Table 2 are phase I or phase II studies which tells about the early state of the investigation of these drugs and further studies are needed if a marketing authorization is in the target. Liposomes complexed with plasmid DNA have been used in the most of the clinical trials with varying indications in Table 2. Biodegrability, low toxicity and non-immunogenicity are properties connected to cationic lipids, which probably leads to their wider investigation compared to polyplexes in clinical trials (Chesnoy and Huang 2000; Tarhini et al. 2011; von der Leyen et al. 2011; Lu et al. 2012; Senzer et al. 2013).

Table 2. The carrier-mediated gene delivery in clinical trials. Carrier-mediated gene delivery clinical trials are collected in the table when their results have been published in 2009–2014.

Carrier	DNA form	Trans-gene	Disease	Phase	Reference
DOTAP/DOPE liposomes labelled with anti-transferrin receptor (SGT-53)	plasmid	p53	Advanced solid tumors	I	Senzer et al. 2013
DOTAP/cholesterol liposomes	plasmid	TUSC2	Lung cancer	I	Lu et al. 2012
D-cholesterol/DOPE liposomes	plasmid	NOS	Restenotic coronary artery lesion	I	von der Leyen et al. 2011
Cholesterol/DOTIM liposomes	plasmid	MnSOD	Lung cancer	I	Tarhini et al. 2011
PEG/PEI/ cholesterol (PPC) lipopolymer (EGEN-001)	plasmid	IL-12	Ovarian cancer	I	Anwer et al. 2010
PPC lipopolymer (EGEN-001)	plasmid	IL-12	Ovarian cancer	I	Anwer et al. 2013
PPC lipopolymer (EGEN-001)	plasmid	IL-12	Ovarian, fallopian tube or primary peritoneal cancer	II	Alvarez et al. 2014
CL22 peptide	plasmid	Melan A, gp100	Melanoma	I / II	Steele et al. 2011

Abbreviation: interleukin-12 (IL-12); manganese superoxide dismutase (MnSOD); nitric oxide synthase (NOS); tumor suppressor p53 (p53) tumor suppressor gene (TUSC2)

As an example of a clinical trial conducted with lipoplexes, the gene therapy to the advanced solid tumors has been investigated using DOTAP/DOPE liposomes (Senzer et al. 2013). The liposomes were labeled with a single-chain antibody fragment of an anti-transferrin receptor to target them to the tumor cells expressing transferrin glycoprotein receptors. The p53 transcription factor gene, that induces cell cycle arrest, was transfected to the cancer cells. The presence of transgene in the tumor was evaluated purifying DNA from the biopsies. Reverse transcription PCR with specific primers for the exogenous p53 gene was used to determine transgene expression in the samples. According to the PCR assessment, transgene was present in the biopsies and thus the lipoplexes reached the target site after intravenous infusion.

Another example of a lipoplex formulation studied in a clinical trial is cholesterol/DOTIM/plasmid lipoplexes that have been studied in combination with chemotherapy and thoracic radiotherapy in lung cancer (Tarhini et al. 2011). In the trial, the target of the study drug was interesting: The plasmid was encoding manganese superoxide dismutase (MnSOD) protein to protect the esophagus from the adverse reactions of chemoradiotherapy by decreasing the expression of inflammatory cytokines in the esophagus of the patient. Because the target cells were located in the esophagus, the study drug was given orally to the patients. Toxicities were not observed after the lipoplex administration. On the other hand, neither detectable amounts of transgene were found in the esophagus.

In addition to lipoplexes, the results of the clinical trial using peptide as a carrier of DNA has been published during the recent years. Steele et al. (2011) have studied a dendritic cell therapeutic cancer vaccine in melanoma using CL22-peptide carrier. CL22 is a peptide with a lysine rich N-terminus that is supposed to be responsible for its good DNA condensation property and a C-terminal influenza nucleoprotein sequence that is believed to comprise an epitope presented on the cells of immunologic defense (Haines et al. 2001). In the clinical trial, dendritic cells were transfected *ex vivo* and the patients were vaccinated with the transfected dendritic cells (Steele et al. 2011). Plasmid complexed with CL22-peptide was encoding melanocyte differentiation antigens, melan A and gp100. Thus, the dendritic cells expressing the antigens were suggested activating T- and B-lymphocytes against the cancer cells in melanoma. During the study the evidence of the immunogenicity of the vaccine was obtained, in addition, the vaccine was concluded to be safe and tolerated.

Lipopolyplex formulation combining PEG, PEI and cholesterol (PPC) complexed with plasmid encoding interleukin-12 (IL-12) has been studied in three clinical trials (Table 2). In phase I trials patients with ovarian cancer were enrolled, while in phase II trial the inclusion criteria were broadened also to the patients with fallopian tube and primary peritoneal cancer. In phase I trials, IL-12 has been shown to increase interferon-γ (IFN-γ) concentrations, which is supposed to lead to the inhibition of the tumor growth (Anwer et al. 2010 and Anwer et al. 2013). Lipopolyplexes were observed to be well-tolerated in the trials. However, in the phase II trial three patient

withdrew from the study because of adverse effects like elevated creatinine values, neutropathy, abdominal pain and anemia (Alvarez et al. 2014). In addition, the study drug showed only limited activity. Thus, the phase II trial with the highest dose level questioned the benefit-risk-ratio of the study drug in ovarian cancer. Considering the formulation of the study drug, the cytotoxicity of PEI is well known from *in vitro* studies, which leads to the possibility that PEI could have had influence on the low tolerability of the study drug (Boussif et al. 1995; Godbey et al. 1999).

7 CONCLUSIONS

Gene delivery encounters barriers both outside and inside the cell and they need to be overcome to achieve the sufficient transfection efficiency in the target cells. Carrier-mediated transfection methods and DNA engineering are studied to develop the efficient gene delivery methods. Polyplexes and lipoplexes are the most investigated non-viral gene delivery vehicles and they have many desirable properties: They can protect DNA from nucleases, facilitate the cell entry and enable the endosomal escape of DNA. Lipopolyplexes are also investigated to combine the benefits of both polymers and lipids to one formulation. DNA engineering aims at achieving long-term transgene expression in the transfected cells by decreasing the bacterial components of plasmid DNA or enabling the endosomal replication in the host cells. The careful characterization and optimization of complexes can enable one to form the gene delivery system with sufficient stability and successful gene expression. Carrier-mediated gene delivery has been investigated in clinical trials, however, these formulations have not yet received any marketing authorization in European Union. Insufficient efficiency and possible toxicity are the main obstacles of carrier-mediated gene delivery systems that need to be overcome to achieve the potential treatment for the disease in interest.

EXPERIMENTAL WORK

8 INTRODUCTION

Gene delivery has been suggested as a treatment for many retinal diseases like age related macular degeneration (AMD) that is the most common cause of blindness in the industrial countries (Fernández-Robredo et al. 2014). It has been estimated that AMD will affect even about 196 million people worldwide in 2020 because the prevalence of AMD is increased in elderly and the population is ageing (Wong et al. 2014). In addition to high age, metabolic, functional, genetic and environmental factors have been observed to have an influence on the chronic disease progression, and two subgroups of AMD, atrophic and exudative, can be distinguished (Strauss 2005; Fernández-Robredo et al. 2014).

At the moment, no curative treatment is available for AMD and the need for new therapies increases all the time (Fernández-Robredo et al. 2014). Moreover, drug administration to the posterior segment of the eye is challenging leading to the situation where many diseases of the back of eye do not have an efficient treatment (Urtti 2006). Despite few intravitreally administered anti-VEGF biologics have been developed, curative therapy for AMD or the other retinal diseases is not yet found (Fernández-Robredo et al. 2014; Solomon et al. 2014). Prolonged action dosage forms and gene delivery are the interest of drug development at the moment because of longer drug administration intervals and better efficiency.

In gene therapy, the disease is treated with exogenously given DNA that is transcribed and translated to produce the therapeutic protein. Gene modifications can be combined with the cell replacement when the cells are transfected with the gene of interest before transplantation (Johnen et al. 2012). Retinal pigment epithelium (RPE) is a possible but demanding target for gene therapy in retinal diseases (Remington 2012; Vellonen et al. 2014). Especially, non-viral transfection is hoped to open new opportunities to deliver genes into RPE cells *in vivo* compared with viral vectors which have many concerns including immunogenicity and low DNA payload

capacity (Kay et al. 2001). To make non-viral gene delivery more efficient and safe, new gene delivery vehicles need to be found and studied *in vitro* and *in vivo*.

8.1 Retinal pigment epithelial cells

The retina is a neural layer that forms the innermost coat of the eye (Figure 1) (Remington 2012). It is located between a choroid and a vitreous. The outermost layer of the retina consists of RPE and it is embryologically derived from the external stratum of the optic cup (Garron 1963). RPE cells form a monolayer which is tightly adherent to the choroid. The shape of these cells is dependent on the site in the retina where they are located varying from hexagonal structure to longer and narrower (Garron 1963; Remington 2012). RPE cells have melanosomes that are pigment granules absorbing light. The pigmentation density is the highest in the macula and in the equator of the retina. The RPE cells have microvilli on the apical site and the cells form tight junctions between each other (Garron 1963).

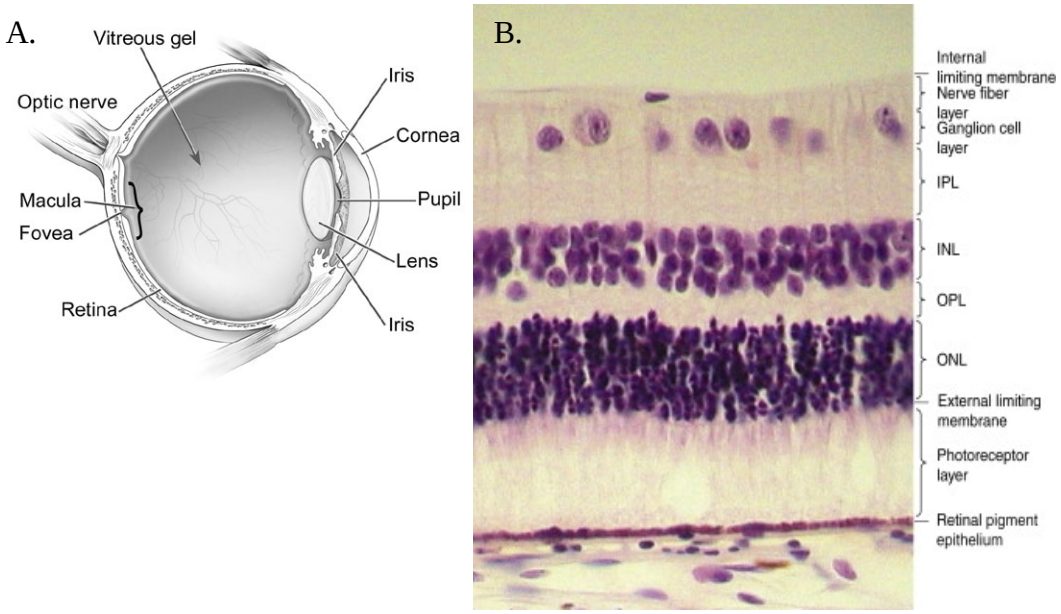

Figure 1. A. Anatomy of the eye. The retina is a neural layer that forms the innermost coat of the eye and it is located between a choroid and a vitreous (National Eye Institute 2014); B. Layers of the retina [outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL)]. A RPE cell layer is the outermost layer of the retina and it is located between photoreceptors and choroid (Remington 2012).

RPE cells have many functions that support the health of photoreceptors (Strauss 2005; Remington 2012). The RPE layer and the endothelial cells of retinal capillaries

form the outer and the inner blood-retina-barrier (BRB), respectively. The outer BRB controls the transfer of nutrients to the photoreceptors and removal of metabolites, ions, and excess of water into the choriocapillaris (Strauss 2005). The RPE is a part of visual cycle which is responsible for the reisomerization of all-*trans*-retinal from photoreceptors to 11-*cis*-retinal and its return to the photoreceptors. RPE cells also have phagocytosis activity, which prevents the accumulation of photo-damaged proteins and lipids in the photoreceptors. The photoreceptors secrete discs that contain light-induced toxic substrates and RPE cells phagocytize and transfer them to the lysosomes. Other functions of RPE cells are to absorb scattered light and produce a variety of growth factors like vascular endothelial growth factor (VEGF) and pigment epithelial derived factor (PEDF) (Steele et al. 1992; Adamis et al. 1993). Furthermore, RPE also maintain the immune privilege of the eye by secreting immunosuppressive factors (Strauss 2005).

Because of many roles of RPE cell layer, its well-being is essential to the normal function of neural retina (Strauss 2005). RPE and photoreceptors can be considered as a functional unit and dysfunction in any task of RPE can lead to the progression of blinding eye diseases. The decreased absorption of light, oxidative stress, diminished phagocytosis and inflammation are examples of processes in the background of AMD. Furthermore, the mutations of genes can promote disease progression. For example, mutation in *ABCA4* transporter gene has impact on the cycle of all-*trans*-retinal and it leads to the different forms of retinal degenerations (Weng et al. 1999). Another example is an imbalance in production of growth factors like VEGF in RPE, which causes the proliferation of the cells (Adamis et al. 1993; Strauss 2005).

8.2 In vitro -cell models for studying transfection to the retina

ARPE-19 is a spontaneously arising, continuous human retinal pigment epithelial cell line that is used in transfection studies (Dunn et al. 1996; Mannermaa et al. 2005; Subrizi et al. 2009). It has highly epithelial morphology, normal karyology and functional properties characteristic of RPE cells, which makes it useful for *in vitro* studies (Dunn et al. 1996). The drawbacks of the cell line are an incorrect phenotype if not grown on the filters and a low melanin formation level (Vellonen et al. 2014). In addition, ARPE-19 subcultures contain both mortal and immortal cells, and

between them can exist differences (Kozlowski 2014). The estimated doubling time for ARPE-19 cells is 24 hours and the passage numbers 25–35 can be used *in vitro* studies (Dunn et al. 1996; Subrizi et al. 2009).

Human embryonic stem cell-derived RPE (hESC-RPE) cells can also be utilized as a cell model of RPE (Vaajasaari et al. 2011; Reynolds and Lamba 2014; ScienCell Research Larboratories 2014). hESC-RPE can be grown for the purposes of cell transplantation or *in vitro* studies (Vaajasaari et al. 2011; Reynolds and Lamba 2014). Embryonic stem cells are derived from the inner cell mass of blastocyst and the differentiation toward RPE can be induced by removing basic fibroblast growth factor and feeder cells from the serum-free conditions. According to the protocol of Vaajasaari et al. (2011), typical pigmented morphology, the expression of RPE-specific markers, secretion of PEDF and tight junctions were achieved after the differentiation of hESC-RPE. hESC-RPE are indicated to resemble nearly human fetal RPE though some differences in gene expression have been found (Liao et al. 2010). Another possibility is to use human embryonic primary RPE cells isolated from the retina of an embryo as a cell model of RPE thought limited availability is related to it. Human primary RPE cells can be cultured for ten doublings *in vitro* but the properties of original tissue can be lost during subculturing (ScienCell Research Larboratories 2014; Vellonen et. al 2014).

The human endothelial hybridoma (EaHy 926) cell line is produced fusing human umbilical vein endothelial cells with a human carcinoma cell line (Edgell et al. 1983). The EaHy 926 cells have been observed to be stable at least till 30 passages (Emeis and Edgell 1988). In this study, endothelial cells are used as a cell model to illustrate the transfection possibilities in choroidal neovascularization. For example, exudative AMD is characterized by the proliferation of abnormal vessels in the choroid which makes endothelial cells a potential target of gene therapy (Fernández-Robredo et al. 2014).

8.3 Non-viral transfection strategies for retinal diseases

Non-viral transfection demands efficient carriers and DNA modifications to compete to viral transfection methods. The prolonged expression of a transgene is needed to

achieve the stable and long-term production of a therapeutic protein in the target cells. The modifications of DNA can have a significant impact on the transfection efficacy and the duration of transgene expression (Darquet et al. 1997). Because minicircles are devoid of bacterial backbone, they are shown to produce higher and longer transgene expression compared with plasmids. On the other hand, plasmid expressing Epstein–Barr virus nuclear antigen 1 (EBNA-1) is supposed to have long transfection efficiency as well because of its episomal replication (Adams 1987). The plasmids with inherent ability to replicate and retain in the nucleus are believed to provide sustained gene expression.

In earlier studies, micelles based on PDMAEMA structure (Alhoranta et al. 2011), DOTAP/DOPE/PS/DNA lipoplexes (Mannermaa et al. 2005) and lipid coated DNA complexes (LCDCs) (Lehtinen et al. 2008) are used to study transfection efficiency *in vitro* with encouraging results. PDMAEMA-based copolymers are observed to have reasonable transfection efficiency in ARPE-19 cells (Alhoranta et al. 2011). DOTAP/DOPE/PS/DNA lipoplexes are also shown to transfect ARPE-19 cells and the transgene has been expressed for two months from the transfection (Mannermaa et al. 2005). On the other hand, LCDCs have not convinced as a gene delivery vehicle in ARPE-19 cells but because of their attractive structure, the composition of LCDCs can be modified for new transfection studies (Subrizi 2004).

Transfection studies with above mentioned carriers complexed with EBNA-1 plasmid or minicircles have not earlier been conducted. Complexing the carriers with the engineered DNA forms is hypothesized to enable an increase in their transfection efficiency and prolonged transgene expression. Thus, transfection studies to RPE and endothelial cells with these complexes is an interesting subject of research that targets to the gene delivery to the posterior eye in the future.

9 AIMS OF THE STUDY

The aims of this experimental work were to investigate new gene delivery systems for the diseases of the posterior eye. Non-viral gene delivery to dividing RPE cells

and endothelial cells was studied *in vitro* using several carrier/DNA combinations. The objectives of this study can be divided into the following points:

1) Characterize and optimize carrier/DNA complexes for transfections.
2) Evaluate and compare the transfection efficiency and the toxicity of PDMAEMA-based complexes, DOTAP/DOPE/PS/DNA lipoplexes and LCDCs with the positive control in RPE and endothelial cells.
3) Evaluate the significance of DNA modifications to the duration and the efficiency of marker protein secretion when episomal plasmid or minicircles are used.
4) Determine the most optimized carrier/DNA complexes to transfect RPE and endothelial cells.

10 MATERIALS AND METHODS

10.1 Materials

1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP, M_w=698,55 g/mol, #890890), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, M_w=744,05 g/mol, #850725) and dioleoylglycerol (diolein, M_w=620,99, #D8894) were used as 10 mg/ml stock solutions in chloroform. 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE, M_w= 691,96, #850705) was dissolved in chloroform/methanol (9:1 v/v) mixture in concentration of 9 mg/ml. The lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) except to diolein that was obtained from Sigma-Aldrich (St. Louis, MO, USA). Branched polyethylenimine (PEI, M_w≈25 000 g/mol, #40872-7) used as 0,9 µg/µl in H_2O pH 7,2, cholesterolhemisuccinate (CHEMS, M_w=486,73 g/mol, #C6512) 10 mg/ml in chloroform, protamine sulfate (PS, M_r≈5000–10 000, #P3369) 1 µg/µl in H_2O and dextran sulphate sodium salt (DS, M_r=40 000, #42867-56) 10 mM in H_2O were also purchased from Sigma-Aldrich. Star-like and linear block copolymers, polystyrene with cationic 2-(dimethylamino)ethyl methacrylate (PS36200-PDMAEMA26400 and PDMAEMA26400-PS36200-PDMAEMA26400; M_w[PS]=36 200 g/mol; M_w[PDMAEMA]=26 400 g/mol) and poly(n-butyl-acrylate) with 2-(dimethylamino)ethyl methacrylate (PBuA4700-PDMAEMA12400 and

PDMAEMA33000-PBuA38700-PDMAEMA33000; M_w[PBuA]=4700 g/mol; M_w[PDMAEMA in linear copolyer]=12 400 g/mol or M_w[PDMAEMA in star-like copolyer]=33 000 g/mol) were synthesized by Anu Alhoranta at the Department of Chemistry, laboratory of polymer chemistry in the University of Helsinki.

10.2 Production of plasmid DNA and minicircles

The secreted alkaline phosphatase (SEAP) marker gene coding plasmid with cytomegalovirus (CMV) promoter and Epstein-Barr virus (EBV) nuclear antigen expression vector (EBNA-CMV-SEAP) was amplified in *Escherichia coli* (*E. coli*). The plasmid was purified by column separation according to the manufacturer's instructions (QIAfilter Plasmid Giga Kit, #12291, Qiagen, USA) and the plasmid was dissolved in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7,4).

The purity of plasmid was enhanced by plasmid DNA precipitation. 0,1 volume of 3 M sodium acetate pH 5,2 at +4°C and 2,5 volumes of absolute alcohol (Aa) at -20°C was added to plasmid in TE-buffer and the solution was vortexed. After incubation at -20 °C for 30 min, the solution was centrifuged at 15 000 g for 30 min at +4 °C. Supernatant was discarded, and the DNA pellet was washed with 1 ml 70 % EtOH at -20 °C inverting the tube a couple of times, after which the centrifugation was repeated for 20 min with the same settings as earlier described. The supernatant was discarded and the DNA pellet was dried in the lyophilizator. After the pellet was dry, DNA was dissolved in TE buffer pH 7,4. The plasmid was stored in TE-buffer at the concentration of 1,91 µg/µl at +4 °C and the concentration of DNA was measured at the wavelength of 260 nm by SPECTROstar Nano (BMG Labtech, Germany).

The plasmid was identified by digestion and electrophoresis. For restriction endonuclease reaction, the regents were added to H_2O in the following order: 2,0 µl of Buffer D, 0,5 µl of bovine serum albumin (BSA) x100, 1 µg of plasmid, and 20 IU of Xbal enzyme and H_2O to 20 µl. The solution was incubated at 37 °C for 2 h and the electrophoresis was conducted in 1 % (w/v) agarose gel with 0,15 µg/µl ethidium bromide at 80 V for 2 h using 1x tris-acetate-ethylene diaminetetraacetic acid (TAE)

buffer. The images of the gel were taken with Bio-Rad ChemiDoc XRS+ imaging system (CA, USA).

The SEAP marker gene coding minicircles with CMV promoter (CMV-SEAP) or human elongation factor-1 alpha promoter (EF1a-SEAP) were produced in engineered *E. coli* bacterial strain using MC-Easy™ System kit (MC-Easy minicircle DNA production kit: [MN920A-1], pMC.BESPX-MCS1 empty vector [MN100A-1], pMC.EF1-MCS-SV40polyA [MN502A-1], System Biosciences, USA). Like plasmid, the minicircles were purified by column separation according to the manufacturer's instructions except to the double volumes of Buffers P1–P3 were used (QIAfilter Plasmid Mega Kit, #12281, Qiagen, USA). The purity of minicircles was enhanced by DNA precipitation like earlier described. The minicircles were stored in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7,4) at the concentration of ~1 µg/µl at +4 °C. The minicircles were identified by digestion and electrophoresis.

10.3 PEI/DNA polyplexes

PEI/DNA complexes with different nitrogen/phosphorus (n/p) ratios were prepared (Subrizi et al. 2009). N/p ratios were calculated in moles supposing that 325 g/mol corresponds one repeating unit of DNA containing one negative charge of phosphate and 43,1 g/mol corresponds one repeating unit of PEI having one positive charge of nitrogen. The total positive charges of the carrier were divided by the negative charges of DNA and several n/p ratios were formed (+1/2, +1, +2, +4, +6, +8, +10 and +16). PEI/DNA complexes were produced by making PEI and DNA dilutions and adding the equal volume of PEI dilution to DNA dilution, both diluted in 10 mM Mes-Hepes buffered saline (50 mM Mes, 50 mM Hepes and 75 mM NaCl in H_2O). When PEI solution at n/p ratio +16 was concerned, 2,1 µg corresponding 49,28 nmol of diluted PEI was mixed with 1 µg corresponding 3,08 nmol of diluted DNA. The solution was mixed twice with a pipette and vortexed for 2 s. The complexes were incubated at room temperature for 2 hours.

10.4 DOTAP/DOPE liposomes and DOTAP/DOPE/PS/DNA lipoplexes

A lipid film hydration method was used to form DOTAP/DOPE (1:1 mol/mol) liposomes (Mannermaa et al. 2005). The production of 0,5 ml of lipid stock solution was started by mixing the volumes corresponding 1,6 mmol of DOTAP and 1,6 mmol of DOPE together. After mixing, the chloroform was dried under the nitrogen and the formed lipid films were further dried overnight in the vacuum desiccator. 0,5 ml of sterile mqH_2O was added on the lipid film to form 3,2 mM stock solution of the lipids and the flask was spun for 30 min at 30 °C in a rotavapor to hydrate the lipid film. The lipids were incubated at room temperature for 2 hours after which they were extruded through 0,1 µm membrane 21 times with Mini-Extruder (Avanti Polar Lipids, AL, USA). The liposomes were stored at ~ +4 °C for a week.

When DOTAP/DOPE/PS/DNA lipoplexes were formed, the equal volumes of DNA and protamine sulfate (PS) dilutions in Mes-Hepes buffer were mixed at the ratio 0,47 (w/w). The PS/DNA mixture was incubated for 10 min and the equal volume of DOTAP/DOPE liposomes diluted in sterile H_2O was added to form lipoplexes at several n/p ratios (+4,45, +6, +8). N/p ratios were calculated in moles supposing that 325 g/mol corresponds one repeating unit of DNA containing one negative charge of phosphate, PS has 21 positive charges per mole and DOTAP/DOPE stock solution has one positive charge per 3,2 mmol. The formed lipoplex solution was incubated for 2 hours.

10.5 Micelle formation and PDMAEMA-based polyplexes

A water addition method was used to produce copolymer micelles (Alhoranta et al. 2011). PBuA-PDMAEMA or PS-PDMAEMA copolymers were dissolved in N-N-dimethylformamide (DMF, ≥99,8%, #319937, Sigma) in the concentration of 5 mg/ml and they were stirred at the room temperature overnight. mqH_2O was added drop wise during 1 hour to form 2 mg/ml solution. The polymer solutions were loaded into the dialysis cells with 12000–14000 Daltons membrane (Medicell International Ltd, London, United Kingdom) and they were dialyzed against 2 liters of mqH_2O to remove DMF changing dialysis H_2O twice a day for two days. On the

third dialysis day, the dialysis buffer was changed to 0,05 M saline to form isotonic micelle solution. The saline buffer was changed twice a day until the micelles were recovered at the end of the fourth dialysis day. The ready micelles were stored at +4 °C for 1 month.

Copolymer/DNA complexes with varying n/p ratios (mol/mol) were formed in Mes-Hepes buffer. The equal volume of copolymer dilution was added to DNA dilution mixing with a pipette, after which the complexes were incubated for 2 hours. It is supposed that 314 g/mol corresponds one repeating unit of cationic PDMAEMA and has one positive charge of nitrogen and 325 g/mol corresponds one repeating unit of DNA containing one negative charge of phosphate. Because positive charges are located in the PDMAEMA part of copolymer, the proportion of PDMAEMA in a copolymer needs to be taken into account when calculating the charges of polyplexes. The proportion of PDMAEMA can be calculated using the molecular weights of blocks in the certain copolymer. For example, concerning linear PDMAEMA-PBuA-PDMAEMA copolymer the portion of PDMAEMA is obtained by the following calculation: $\frac{2*MW(PDMAEMA)}{2*MW(PDMAEMA)+MW(PBuA)}$.

10.6 Lipid coated DNA complexes

DOPE/CHEMS (3:2 mol/mol), diolein/CHEMS (3:2 mol/mol) and DPPE/CHEMS (3:2 mol/mol) coated complexes were prepared according to Lehtinen et al. (2008). PEI/DNA complexes were produced mixing equal volumes of diluted PEI and DNA solutions in 10 mM HEPES (pH 7,4) together. N/p ratios +2, +4, +6 and +8 were used with the DNA concentration of ~48 µg/µl. The lipid shields were formed by a detergent removal method. The total lipid content was adjusted to 1,3 µmol (half lipid), 2,6 µmol (full lipid) or 5,2 µmol (twice lipid) per a tube. Desired volumes of lipid solutions were measured to a glass tube and chloroform/methanol was dried under the nitrogen and in a vacuum desiccator overnight.

The lipid film was dissolved with 5 % octylglucoside (OG) solution in H_2O (m/v) by vortexing. The volumes corresponding 8 µg, 10 µg and 11,5 µg of OG were added on half, full and twice lipid, respectively. The mixture was warmed to 60 °C in the

water bath if the lipid film did not dissolve at room temperature. The volumes of 17,5 µl, 22 µl and 25,5 µl of 100 mM Hepes pH 7,4 was added to half, full and twice lipid, respectively. 10 mM Hepes, pH 7,4 was added to have a volume of 500 µl of each lipid. The equal volume of PEI/DNA complexes were added drop wise into the lipid solution stirring with a magnetic stirrer at 250 rpm. After that the solution was diluted with 1000 µl of 10 mM Hepes drop by drop. The solution was loaded into dialysis cassettes (Slide-A-Lyzer Dialysis Cassette 10000 MWCO, #66380, Thermo Scientific, MA, USA) and dialyzed stirring at 250 rpm against 2 liters of 10 mM Hepes at +4 °C overnight changing the dialysis buffer after 1, 3 and 6 hours. The ready lipid coated DNA complexes (LCDCs) were recovered on the next day.

10.7 Size measurement and zeta potential

The size and zetapotential of the complexes was measured by dynamic light scattering. The complexes were produced like earlier described. For zeta potential measurement, the complexes were formed in 10 mM Hepes because of the suitable conductivity of the buffer for the measurement. Zetasizer APS (Malvern Instruments, United Kingdom) was used to study particle size and Zetasizer Nano (Malvern Instruments, United Kingdom) zetapotential. The setup and analysis were carried out by Malverns Zetasizer Software V.7.02.

10.8 Gel retardation assay

The complex formation efficiency of nanoparticles was studied by electrophoresis on agarose gel. 200–600 ng of DNA was used per well depending on the well size of the comb. In order to evaluate the stability of complexes, three times excess in moles of negatively charged dextran sulfate (DS) was added to the complex dilution and the mixtures were incubated for 1 h before electrophoresis. For non-treated samples the same volume of Mes-Hepes or 10 mM Hepes buffer was added. Electrophoresis was conducted in 1 % (w/v) agarose gel with 0,15 µg/µl ethidium bromide (EtBr) at 80 V for 40–120 min using TAE buffer. The images of the gel were taken with Bio-Rad ChemiDoc XRS+ imaging system (CA, USA).

10.9 DNA relaxation assay

The condensation efficiency and stability of complexes were determined by DNA relaxation assay. The complexes were produced like described earlier. 150 µl of the complex solution corresponding 1 µg of DNA or 1 µg of free DNA as a control was used per well in triplicates in a black 96-well plate. 50 µl of EtBr dilution (10 µg/µl) was added per well. The fluorescence was measured at the excitation wavelength of 518 nm and the emission wavelength of 605 nm with Varioskan™ Flash Multimode Reader 4.00.53 (Thermo Scientific, MA, USA), after which three times excess (mol/mol) of dextran sulfate (50 µl) was added. The fluorescence was measured immediately, after 20 min, 60 min and 24 h. After the last measurement, 50 µl of non-ionic surfactant Triton X-100 (#807423, MP Biomedicals LLC) 10 % in H_2O was added to wells containing LCDCs to disintegrate the complexes and the fluorescence was measured immediately, after 20 min, 60 min and 24 h. The fluorescence of the samples was divided by the fluorescence of free DNA to get relative fluorescence values.

10.10 Cell cultures

ARPE-19 cells were cultured in DMEM/F12 (#31330, Gibco) supplemented with 10 % (v/v) of fetal bovine serum (FBS), 1 % (v/v) of 100x GlutaMAX supplement (#35050-038, Gibco) and 1 % (v/v) Pen-Strep. The cell culture was grown at 37 °C in 7 % CO_2 atmosphere. The cells were subcultured once a week. For transfection, 20 000 cells were seeded per well on a 96-well plate (Nunc™ MicroWell™ Plates with Nunclon™ Delta Surface, #163320, Nunc). The passage number 29 was used in transfections.

Human embryonic stem cell-derived retinal pigment epithelial cells were received from Heli Skottman, Ophthalmology group, Institute of Biomedical Technology, the University of Tampere. The cells were cultured in KnockOut DMEM (#10829-018, Gibco) supplemented with 15 % of KnockOut Serum Replacement (#10828-028, Gibco), 1% of 100x GlutaMAX supplement (#35050-038, Gibco), 1% of 100x NEAA (#BE13-114E, Biowhittaker), 0,2 % of 50 mM 2-Mercaptoethanol (#31350-010, Gibco) and 50 U/ml of Pen-Strep (#17-602E, Biowhittaker) on 24-well or

48-well plate. The medium was changed three times a week. The cells were incubated at 37 °C in 5% CO_2 atmosphere (HERAcell 150 CO_2 incubator, Thermo Electron Corporation, Vaasa, Finland). For transfection, 200 000 cells were seeded to a well on 48-well plate using the cell passages 46 and 47 (Corning® CellBIND® cell culture multiwell plate, #3338, Sigma-Aldrich).

Human embryonic primary retinal pigment epithelial cells (HRPEpI, 6540) were obtained from ScienCell. The flasks were coated with poly-L-lysine (#0403, SciCell) 0,2 or 2 µg/cm^2 a day before the cell seeding. The cells were cultured in Epithelial Cell Medium with 2 % of FBS (#0010, SciCell), 1 % of epithelial cell growth supplement (#4152, SciCell) and 1 % of penicillin/streptomycin solution (#0503, SciCell). For transfection, 50 000 cells were seeded per well on poly-L-lysine coated (2 µg/cm^2) 48-well plates (Corning® CellBIND® cell culture multiwell plate, #3338, Sigma-Aldrich).

Human endothelial hybrid cell line (EaHy 926) was cultured in DMEM (#41966-029, Gibco) supplemented with FBS (#10270, Gibco), 1% of 100x GlutaMAX supplement (#35050-038, Gibco) and 1 % of Pen-Strep (#17-602E, Biowhittaker). 200 µl of 50x HAT supplement (#21060-017, Gibco) was added to 10 ml of medium just before medium change. The cells were subcultured twice a week. For transfection, 20 000 cells were seeded per well on a 48-well plate (Nunclon Surface 48-well plate, #150687, Nunc). The cell passages 19 and 27 were used for transfections.

10.11 Transfections

hESC-RPE, primary RPE and EaHy cells were seeded on 48-well plates and ARPE-19 cells on 96-well plates a day before transfection. Transfection was conducted according to the protocol optimized by Subrizi et al. (2009). The complexes were prepared and incubated for two hours, after which they were added on the cell cultures. For ARPE-19 cells 0,6 µg of DNA and to hESC-RPE, HRPEpi and EaHy cells 0,8 µg of DNA was used per well. The cells were incubated with complexes in serum-free medium for 2 h at 37 °C. After that the cells were washed

with D-PBS and 150 µl (ARPE-19), 200 µl (hESC-RPE) or 250 µl (EaHy and Human primary RPE cells) of full medium was added per well.

10.12 Evaluation of the transfection efficiency

The first samples for SEAP secretion analysis were collected on day 1 after the transfection and the sample taking was continued once a day extending the sample taking interval during the time. The sample taking was stopped once the SEAP secretion level decreased significantly or the viability of the cells lowered. The cell growing medium was collected at certain time points and the samples were centrifuged at 12 000 g for 30 s or 2750 g for 1 min to remove possible detached cells. The collected medium was stored at -20 °C before analysis. The samples were analyzed with Great EscAPe™ SEAP Chemiluminescence Kit 2,0 (#631738, Clontech, USA) according to the manufacturer's instructions. Briefly, 25 µl of removed medium was transferred to a luminescence plate and 75 µl of dilution buffer was added. In order to remove the impact of natural phosphatase enzymes, the samples were incubated at 65 °C for 30 min. 100 µl of SEAP substrate was added per well and samples were incubated at room temperature ~30 min after which chemiluminescence was measured with Varioskan™ Flash Multimode Reader 4.00.53 (Thermo Scientific, MA, USA). The standard curve of SEAP positive control was produced to convert relative light units to the quantity of SEAP. The standard curve covered the SEAP concetrations 0,00096–2 ng/µl. If the concentration of SEAP was not within the standard curve, the SEAP samples were diluted with the dilution buffer and the samples were analyzed again like described above. As a negative control, medium from non-transfected cells was used. The luminescence value from the full medium was used to evaluate the background effect.

10.13 Evaluation of the cell viability

The cell viability was estimated based on the microscope pictures and using AlamarBlue test. In AlamarBlue test fresh medium was added to the cells and 10 % of alamarBlue™ Dye (#DAL1100, Thermo Scientific) was added to the medium. The cells were incubated at 37 °C for 2–4 h and the medium was collected into a black 96-well plate. The fluorescence was measured at the excitation wavelength 540

nm and the emission wavelength 590 nm with VarioSkan. The relative fluorescence was calculated by dividing the obtained fluorescence values of the medium from the transfected cells by the fluorescence value of the medium collected from the wells of the cells that were not transfected.

11 RESULTS

11.1 Size and zetapotential measurements

The size and zetapotential of nanoparticles complexed with plasmid (EBNA-CMV-SEAP) or minicircle (CMV-SEAP or EF1a-SEAP) are collected in Table 1. Several n/p-ratios were used to form nanoparticles for the measurements.

Table 1. Z-average size (±standard error of the mean, SEM), polydispersity index (PdI) and zetapotential (ZP) (±SEM) of carrier/DNA complexes. The Z-average size was measured in Mes-Hepes except to the size of LCDCs was measured in 10 mM Hepes. The setapotential of all the complexes was measured in 10 mM Hepes.

Carrier/DNA complex	Z-Average (nm)	SEM (±nm)	PdI	n (size)	ZP (mV)	SEM (±mV)	n (ZP)
Plasmid (EBNA-CMV-SEAP)							
PEI +4	458	219	0.499	2			
PEI +6	181	17	0.318	7	40.0	0.4	2
PEI +8	167	16	0.389	6	40.0	0.1	2
PEI +10	162	16	0.380	2	-	-	-
DOTAP/DOPE/PS +4,45	203	38	0.470	4	37.2	2.8	2
DOTAP/DOPE/PS +6	130	0	0.095	2	-	-	-
DOTAP/DOPE/PS +8	136	6	0.138	2	-	-	-
PS-PDMAEMA linear +2	209	9	0.218	4	-	-	-
PS-PDMAEMA linear +4	182	9	0.230	5	32.6	0.7	2
PS-PDMAEMA linear +8	142	-	0.205	1	-	-	-
PS-PDMAEMA linear +16	131	-	0.170	1	-	-	-
PS-PDMAEMA star +2	427	-	0.219	1	34.1	0.4	2
PS-PDMAEMA star +4	507	-	0.246	1	-	-	-
PS-PDMAEMA star +8	493	-	0.189	1	-	-	-
PS-PDMAEMA star +16	533	-	0.200	1	-	-	-
PBuA-PDMAEMA linear +2	170	8	0.277	4	-	-	-
PBuA-PDMAEMA linear +4	145	2	0.250	3	-	-	-
PBuA-PDMAEMA linear +8	125	-	0.197	1	-	-	-
PBuA-PDMAEMA linear +16	120	-	0.208	1	-	-	-
PBuA-PDMAEMA star +2	214	8	0.307	4	33.6	0.2	2
PBuA-PDMAEMA star +4	195	2	0.281	3	-	-	-
PBuA-PDMAEMA star +8	177	-	0.438	1	-	-	-
PBuA-PDMAEMA star +16	200	-	0.431	1	-	-	-
DreamFect	199	-	0.277	1	-	-	-
DOPE/CHEMS Half lipid PEI +6	213	33	0.352	3	-55.4	2.1	2
DOPE/CHEMS Half lipid PEI +2	287	-	0.255	1	-	-	-
DOPE/CHEMS Full lipid PEI +6	176	-	0.336	1	-58.6	1.6	2
DOPE/CHEMS Full lipid PEI +4	151	-	0.353	1	-	-	-

Table 1 continues:

DOPE/CHEMS Twice lipid PEI+8	96	3	0.420	2	-62.9	-	-
Diolein/CHEMS Half lipid PEI +6	179	25	0.295	4	-67.1	-	-
Diolein/CHEMS Half lipid PEI +8	145	-	0.227	1	-	-	-
Diolein/CHEMS Full lipid PEI +6	175	10	0.289	2	-71.5	5.1	2
Diolein/CHEMS Full lipid PEI +8	139	-	0.277	1	-	-	-
DPPE/CHEMS Full lipid PEI +6	433	1	0.480	2	-56.3	-	1
DPPE/CHEMS Twice lipid PEI +6	800	83	0.483	2	-77.0	-	1
Minicircle CMV-SEAP							
PEI +6	272	83	0.356	2	-	-	-
PEI +8	210	14	0.437	2	-	-	-
PEI +10	172	-	0.315	1	-	-	-
DOTAP/DOPE/PS +4.45	216	44	0.459	3	-	-	-
PS-PDMAEMA linear +4	239	32	0.292	2	-	-	-
PBuA-PDMAEMA linear +2	260	5	0.395	2	-	-	-
PBuA-PDMAEMA star +2	238	29	0.359	2	-	-	-
DreamFect	211	-	0.381	1	-	-	-
Minicircle EF1a-SEAP							
PEI+6	701	521	0.574	2	-	-	-
PEI+8	250	50	0.346	2	-	-	-
PEI+10	192	-	0.342	1	-	-	-
DOTAP/DOPE/PS +4.45	184	15	0.423	3	-	-	-
PS-PDMAEMA linear +4	245	14	0.317	2	-	-	-
PBuA-PDMAEMA linear +2	204	17	0.312	2	-	-	-
PBuA-PDMAEMA star +2	247	15	0.347	2	-	-	-
DreamFect	359	-	0.322	1	-	-	-

11.2 Gel retardation assay

The condensation efficiency of carrier/DNA complexes was studied by the electrophoresis on the agarose gel at several n/p ratios. The binding of free DNA to EtBr leads to visible bands on the gel. The complexes were also run with anionic DS to study the relaxation of DNA from the complexes.

As shown in Figure 2, 3 and 4, on the most of the lanes no bands are seen on the gel and the complexes retard DNA completely when DS is not added. The exceptions are micelle/plasmid complexes formed from star-like PBua-PDMAEMA copolymer at n/p ratios +4 and +16. The bands can be observed on the gel although DS is not present (Figure 2, Lanes 16 and 17). Concerning LCDCs coated with diolein/CHEMS or DOPE/CHEMS, faint bands are present at the end of the lanes although DS is not added to the complexes (Figure 2, Lane 9; Figure 3 A, Lanes 3, 5 and 6).

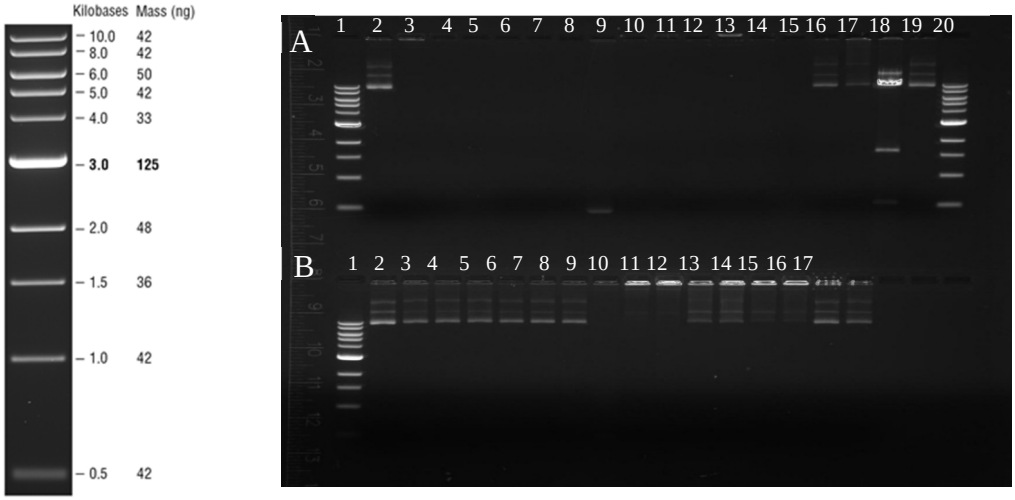

Figure 2. Agarose gel electrophoresis of carrier/DNA complexes with plasmid EBNA-CMV-SEAP. On the row A complexes without DS and on the row B the complexes with DS are presented. On the lane 2 and 19 the uncut plasmid was run and three bands can be observed that are supposed to be multimer, opened and supercoiled plasmid from above to downwards. On the lane 18, the plasmid digested by Xbal endonuclease is shown.

1. and 20. Ladder
2. and 19. Uncut plasmid (EBNA-CMV-SEAP)
3. PEI +4
4. PEI +6
5. PEI +8
6. DOTAP/DOPE/PS +4,45
7. DOTAP/DOPE/PS +6
8. DOTAP/DOPE/PS +8
9. DOPE/CHEMS twice lipid PEI +8
10. PS linear +4
11. PS linear +16
12. PS star +4
13. PS star +16
14. PBuA linear +4
15. PBuA linear +16
16. PBuA star +4
17. PBuA star +16

The bands are observed on the lanes when PEI/DNA polyplexes, DOTAP/DOPE/PS/DNA lipoplexes and copolymer/DNA complexes formed from star-like PS-PDMAEMA or star-like PBuA-PDMAEMA copolymers are run with DS (Figure 1). Concerning PS-PDMAEMA- and PBuA-PDMAEMA-based polyplexes formed from linear copolymers, only faint bands can be detected on the gel thought DS is present. However, minicircles formed more resistant complexes to DS when they were complexed with star-like PBuA-PDMAEMA copolymer compared with linear PBuA-PDMAEMA copolymer (Figure 4). No bands can be observed on the gel when LCDCs are run on the gel and DS is present (Figure 3).

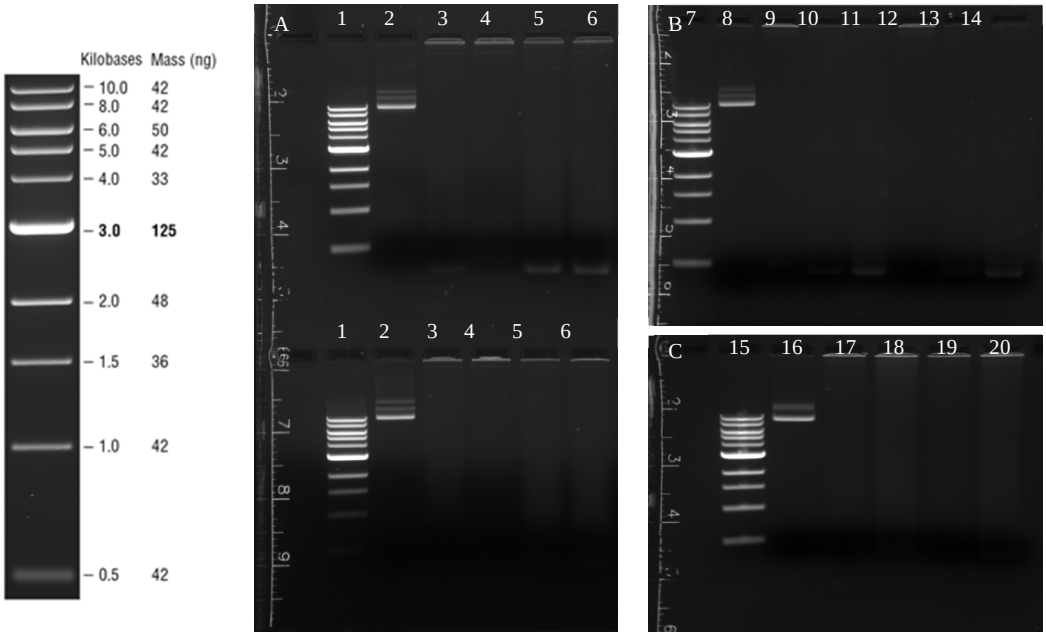

Figure 3. Electrophoresis of LCDCs on agarose gel.
A. diolein/CHEMS coated complexes:
1. ladder, 2. circular plasmid, 3. half lipid PEI +6, 4. half lipid PEI +8, 5. full lipid PEI +6, 6. full lipid PEI +8. On the first row complexes without DS and on the second row with DS are presented.
B. DOPE/CHEMS coated complexes:
7. ladder, 8. circular plasmid, 9. half lipid PEI +6, 10. full lipid PEI +6, 11. twice lipid PEI +8 12. half lipid PEI +6 with DS, 13. full lipid PEI +6 with DS, 14. twice lipid PEI +8 with DS.
C. DPPE/CHEMS coated complexes:
15. ladder, 16. circular plasmid, 17. full lipid PEI +6, 18. twice lipid PEI +6, 19. full lipid PEI +6 with DS, 20. twice lipid PEI +6 with DS.

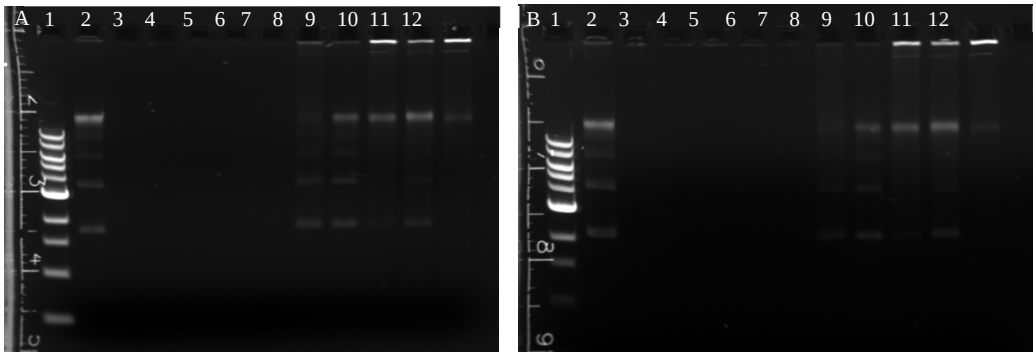

Figure 4. Electrophoresis of carrier/minicircle complexes on agarose gel.
A. Minicircle CMV-SEAP, **B.** Minicircle EF1a-SEAP.
The order of the samples: 1. ladder 2. circular minicircle, 3. PEI +8, 4. DOTAP/DOPE/PS +4,45, 5. PS linear +4, 6. PBuA linear +2, 7. PBuA star +2, 8. PEI +8 with DS, 9. DOTAP/DOPE +4,45 with DS, 10. PS linear +4 with DS, 11. PBuA linear +2 with DS, 12. PBuA star +2 with DS.

11.3 DNA relaxation assay

The release of DNA from the complexes was studied by DNA relaxation assay. PEI/DNA complexes, DOTAP/DOPE/PS/DNA lipoplexes and copolymer/DNA polyplexes disintegrated immediately after DS addition, which is shown in the increase in the relative fluorescence of the samples from 6–40 % to 60–100 % (Figure 5). As shown in Figure 5, PEI/DNA polyplexes at n/p ratio +4 did not complex DNA unlike PEI at higher n/p ratios +6 and +8. The relative fluorescence of PEI/DNA complexes at n/p ratio +4 was 75 % already before DS addition. Copolymer/DNA polyplexes formed from star-like PBuA-PDMAEMA also produced only loose complexes which is shown as ~50 % starting relative fluorescence value. However, the deviation of relative fluorescence between the complexes formed from several star-like PBuA-PDMAEMA batches was high (data not shown).

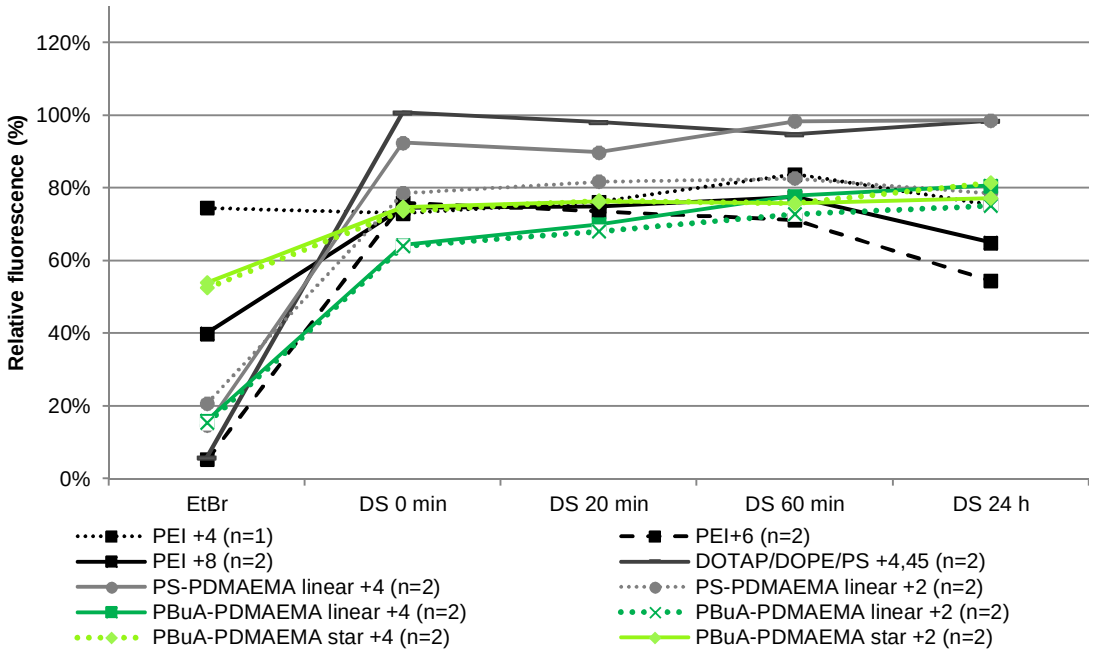

Figure 5. DNA relaxation assay of PEI/DNA complexes, DOTAP/DOPE/PS/DNA lipoplexes and PDMAEMA-based polyplexes. The fluorescence value of complexes was divided by the fluorescence of free DNA. The fluorescence was measured without DS and immediately, after 20 min, 60 min and 24 h of DS addition.

LCDCs are stable against the effects of DS and relative fluorescence remains almost the same regardless of the addition of DS (Figure 6). However, the relative

fluorescence values at the first measuring point vary from 19 % to 45 % between the LCDCs. Non-ionic surfactant Triton X-100 was added to disintegrate the complexes but the detection of a fluorescence signal turned out difficult. Triton X-100 had influence on the background fluorescence value which disturbed the measurement (the data not shown).

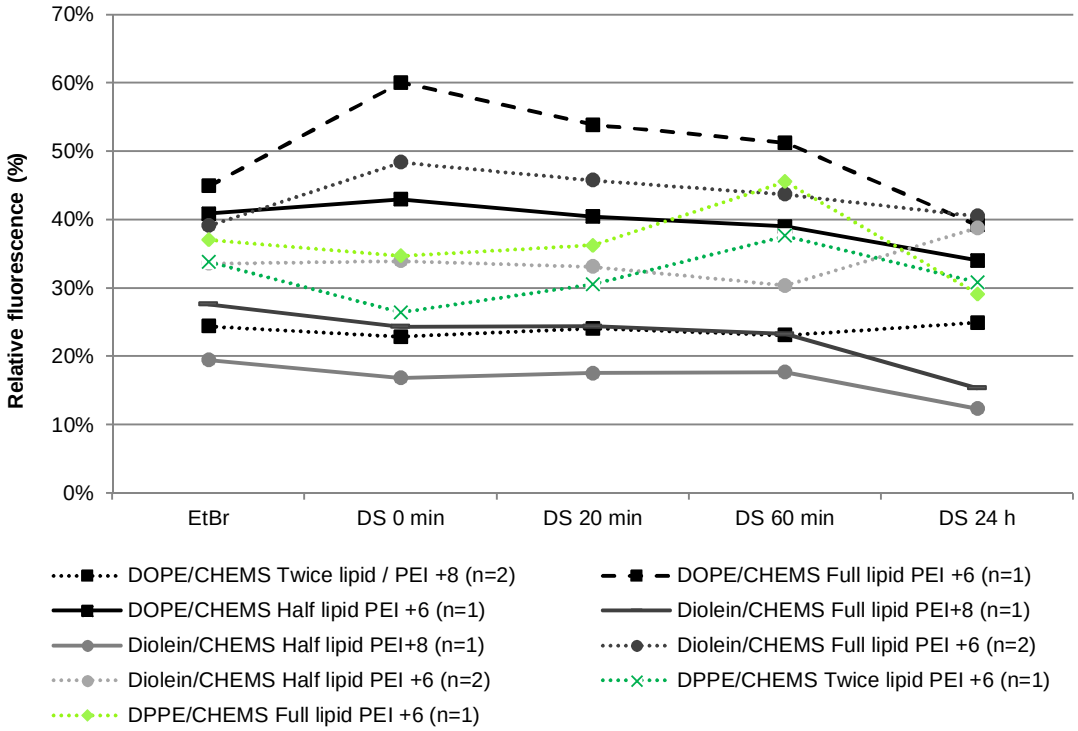

Figure 6. DNA relaxation assay of LCDCs. The fluorescence of complexes was divided by the fluorescence of free DNA. Fluorescence was measured without DS and immediately, after 20 min, 60 min and 24 h of DS addition.

11.4 Transfections

The transfection efficiency of carrier/DNA complexes was evaluated in ARPE-19 cells, hESC-RPE, human embryonic primary RPE cells and EaHy cells. PEI polymer and commercially available DreamFect transfection agent were used as positive control carriers in transfections. The viability of cells was evaluated by microscope observing and with an AlamarBlue test after transfections.

11.4.1 Cell cultures

The viability of cells was observed with the microscope. The pictures of cells were taken before and after transfections (Figure 7). The differences in cell density can be

observed before the transfection to EaHy cells when passage numbers 19 and 27 are compared (Figure 7, Pictures D. and G.).

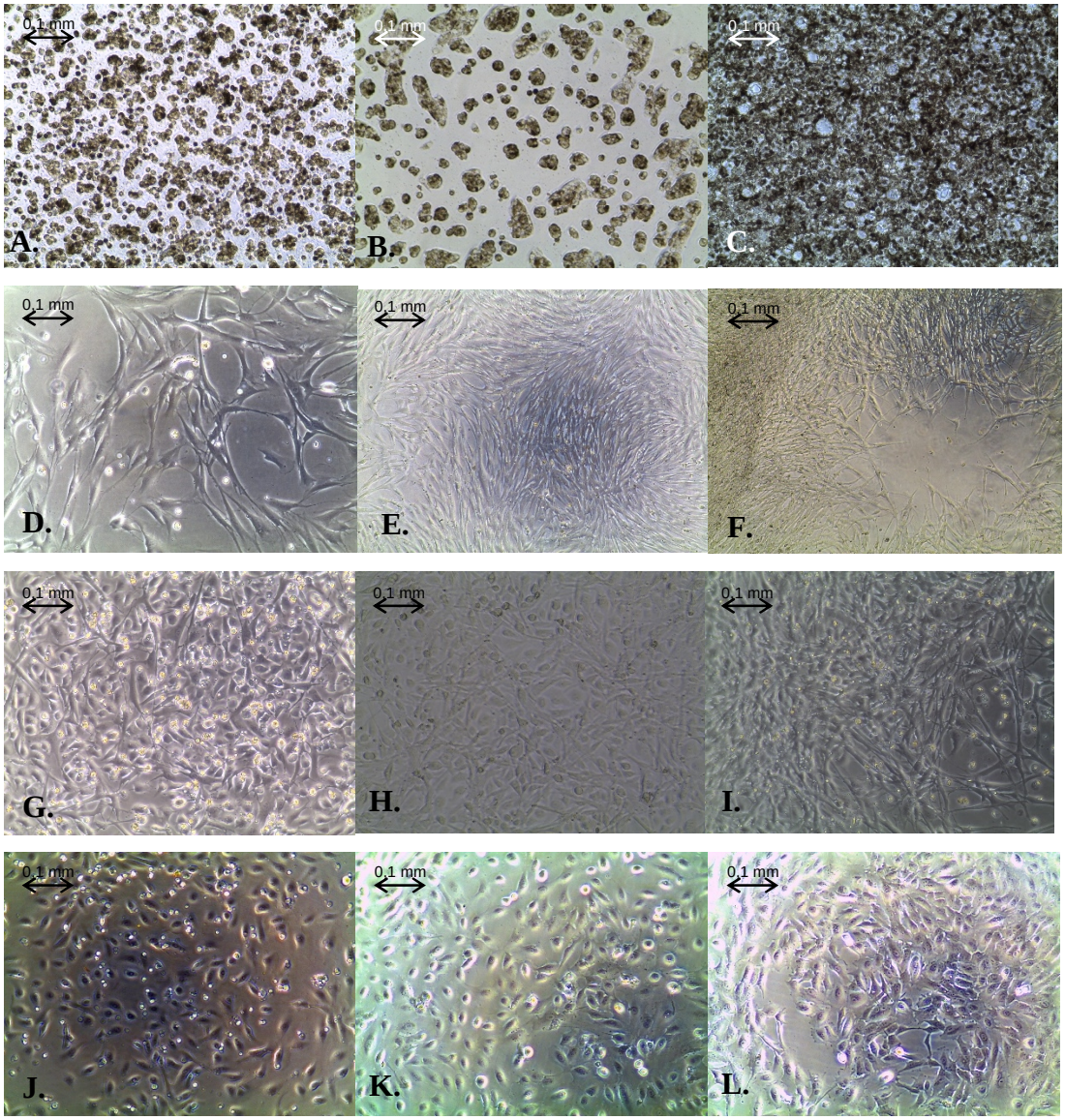

Figure 7. The microscopic pictures (10x) of cells on 48-well plates before and after transfection. Pictures on day 0 have been taken just before transfection.

hESC-RPE / PBuA-PDMAEMA star +2 (plasmid), seeding density 200 000 cells/well, passage number (p.) 46:
A. Day 0, **B.** Day 1 **C.** Day 24.
EaHy / DOTAP/DOPE/PS (plasmid) +4,45, 20 000 cells/well, p. 19:
D. Day 0, **E.** Day 1, **F.** Day 4
EaHy / DOTAP/DOPE/PS (minicircle CMV-SEAP), 20 000 cells/well, p. 27:
G. Day 1, **H.** Day 4, **I.** Day 6
Human primary RPE cells / PBuA-PDMAEMA star +2 (plasmid), 50 000 cells/well, p. 1:
J. Day 0, **K.** Day 1, **L.** Day 3

11.4.2 Transfection of ARPE-19 cells

PEI/DNA complexes at n/p ratios +4, +6 and +8, DOTAP/DOPE/PS/DNA lipoplexes at n/p ratios +4,45, +6 and +8, star-like and linear PS-PDMAEMA/DNA and PBuA-PDMAEMA/DNA polyplexes at n/p ratios from +2 to +16 were used in transfections to dividing ARPE-19 cells. Plasmid DNA EBNA-CMV-SEAP was used to form all the complexes.

The SEAP secretion was the highest on day 2 or 3 after transfection depending on the complex (Figure 8). The cells were followed for 6 days after the transfection. The maximum SEAP secretion rate varied from 0–451 ng/h/ml. PBuA-PDMAEMA/DNA polyplexes from linear copolymer at n/p +2 were the only complexes that reached the transfection efficiency of PEI-complexes. The complexes that did not reach any transfection efficiency are not presented in Figure 8.

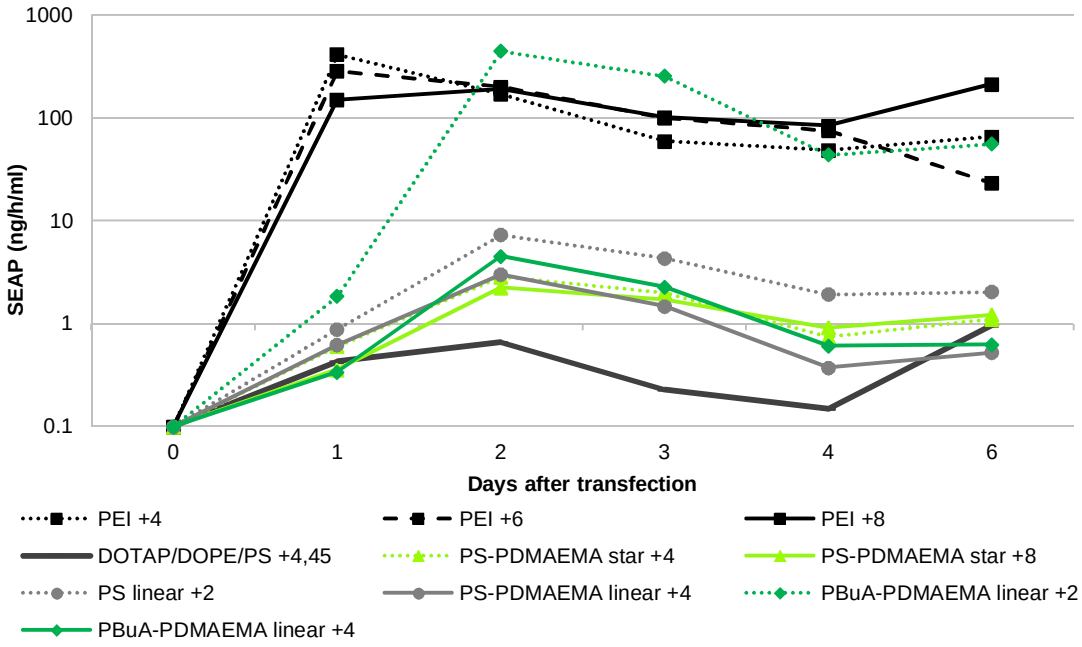

Figure 8. The SEAP secretion rate on the logarithmic scale after transfection to ARPE-19 cells (n=1). Plasmid DNA is used in all the complexes.

According to the data from the AlamarBlue test, the viability of ARPE-19 cells varied widely depending on the complex used (Figure 9). On the day 1 after the transfection, the lowest viability 7 % and 12 % was observed in the wells that were treated with star-like PS-PDMAEMA -based polyplexes at n/p ratio +8 and +16,

respectively. PDMAEMA/DNA polyplexes at higher n/p ratios can be found to decrease more the viability compared with the other complexes and the viability of the cells transfected with PDMAEMA/DNA polyplexes did not always increase during the time after transfection.

It can be shown the dependency on the viability between different n/p ratios in the cells that have been treated with star-like or linear PS-PDMAEMA -based or linear PBuA-PDMAEMA -based polyplexes. The viability is decreasing when the n/p ratio is increasing (Figure 9). The cells transfected with the other complexes do not show such an effect. The metabolic activity of the cells treated with PEI/DNA complexes decreased on day 1 after transfection (21–71 % depending on the n/p ratio). However, the cells reached fast the viability over 100 %. The viability of the cells did not decrease under 80 % when the cells were transfected with DOTAP/DOPE/PS/DNA lipoplexes.

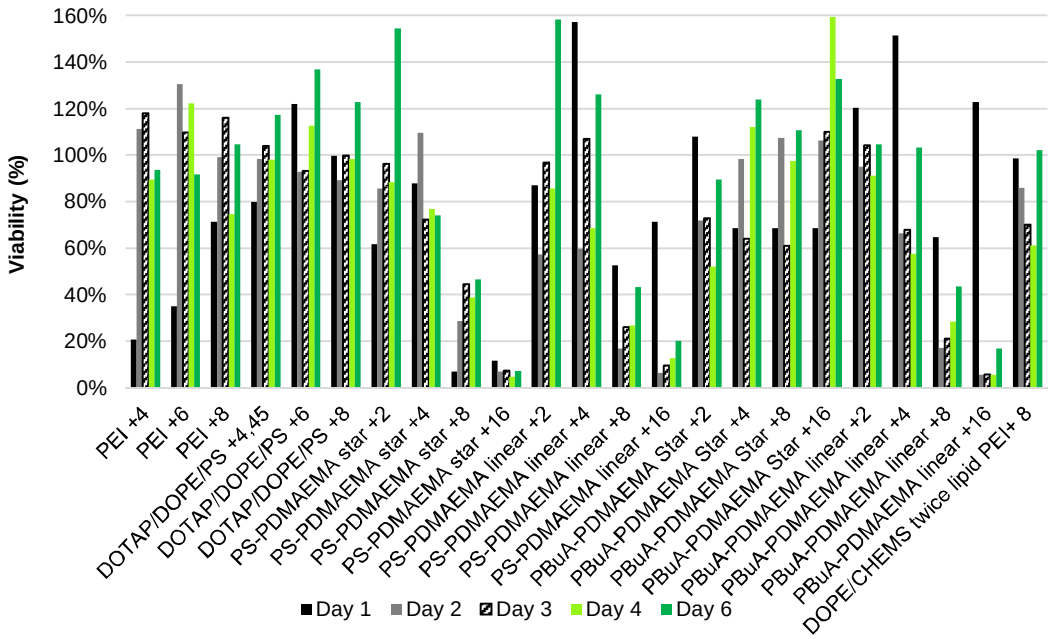

Figure 9. The viability of ARPE-19 cells after transfection (n=1). Plasmid EBNA-CMV-SEAP is used to form all the complexes. The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

11.4.3 Transfection of human embryonic primary RPE cells

Two parallel transfections were conducted to human embryonic RPE primary cells. The transfections were done at the same time and the cells had the same passage

number 1. PEI/DNA complexes at n/p ratio +6 and +8, copolymer/DNA polyplexes formed from linear PS-PDMAEMA, linear PBuA-PDMAEMA and star-like PBuA-PDMAEMA at n/p ratios +2 and +4 and DOTAP/DOPE/PS/DNA lipoplexes at n/p ratio +4,45 were formed with plasmid (EBNA-CMV-SEAP) or minicircle (CMV-SEAP or EF1a-SEAP) for the transfections. The data from transfections with carrier/plasmid complexes are shown in Figure 12 and with carrier/minicircle complexes in Figure 13. LCDCs, linear PS-PDMAEMA complexed with plasmid at n/p ratio +2, DreamFect/plasmid complexes, PEI/EF1a-SEAP minicircle polyplexes at n/p ratio +6 or +8 and PEI/CMV-SEAP minicircle polyplexes at n/p ratio +6 did not have any transfection efficiency. The cells were followed for 18 days.

In human embryonic RPE primary cells, star-like PBuA-PDMAEMA carrier had the highest transfection efficiency and the production of SEAP reached ~35 ng/h/ml and ~47 ng/h/ml when plasmid and CMV-SEAP minicircle were used in the complex with the polymer, respectively. Also the other carrier/plasmid complexes presented in Figure 12 reached the transfection efficiency of PEI/DNA polyplexes. Concerning minicircles, carrier/CMV-SEAP complexes produced higher transfection efficiency compared with carrier/EF1a-SEAP complexes.

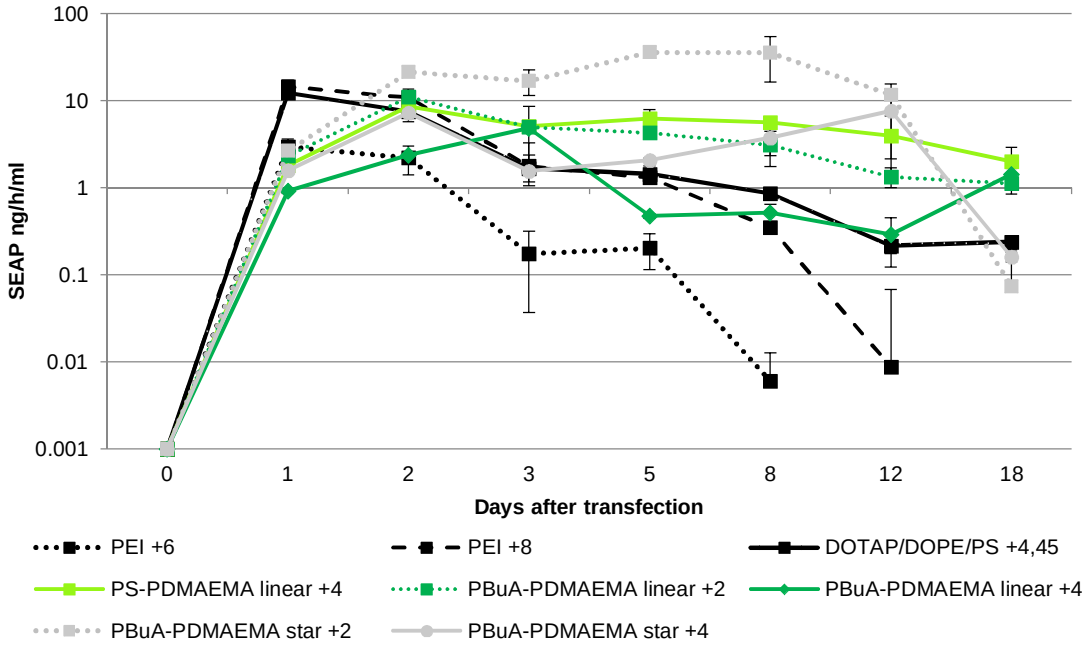

Figure 12. SEAP secretion rate (ng/h/ml ±SEM) on the logarithmic scale after transfection to human primary RPE cells with carrier/plasmid complexes (n=2).

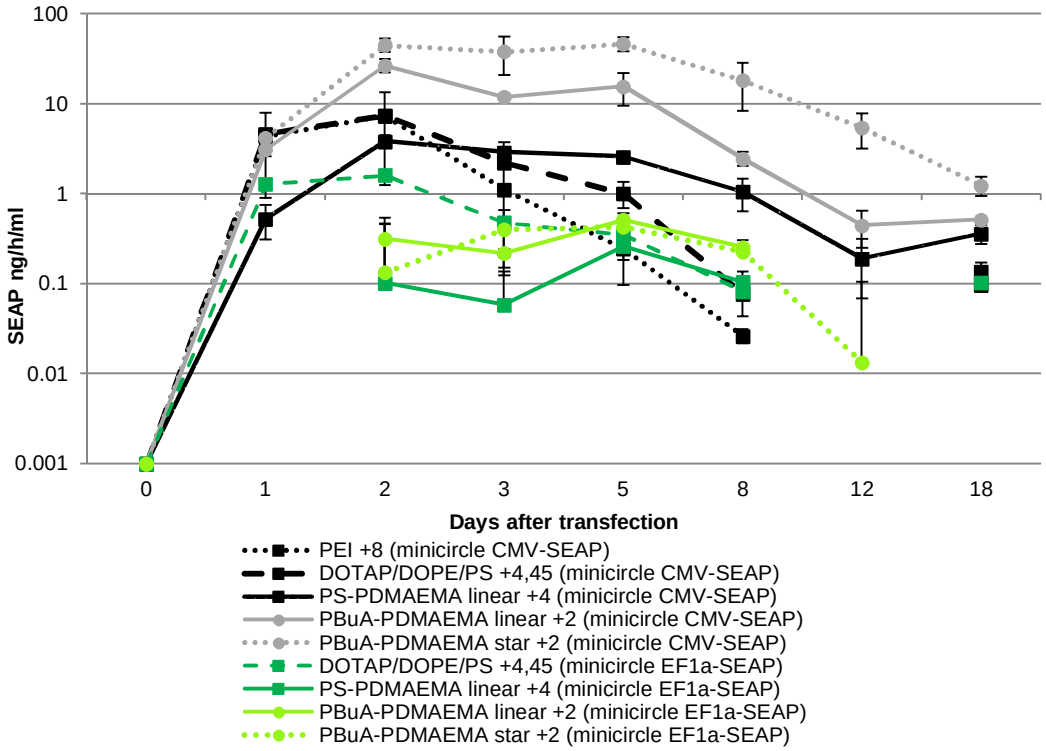

Figure 13. SEAP secretion rate (ng/h/ml ±SEM) on the logarithmic scale after transfection to human primary RPE cells with carrier/minicircle complexes (n=2).

The viability of primary RPE cells was followed for five days after the transfections using the AlamarBlue test. Generally, the metabolic activity stayed over 75 % after the transfection. Star-like PBuA-PDMAEMA -based complexes showed to decrease viability to ~60 % when used at n/p ratio +4. However, the cells recovered from the transfections reaching the viability over 90 % within 5 days from the transfection.

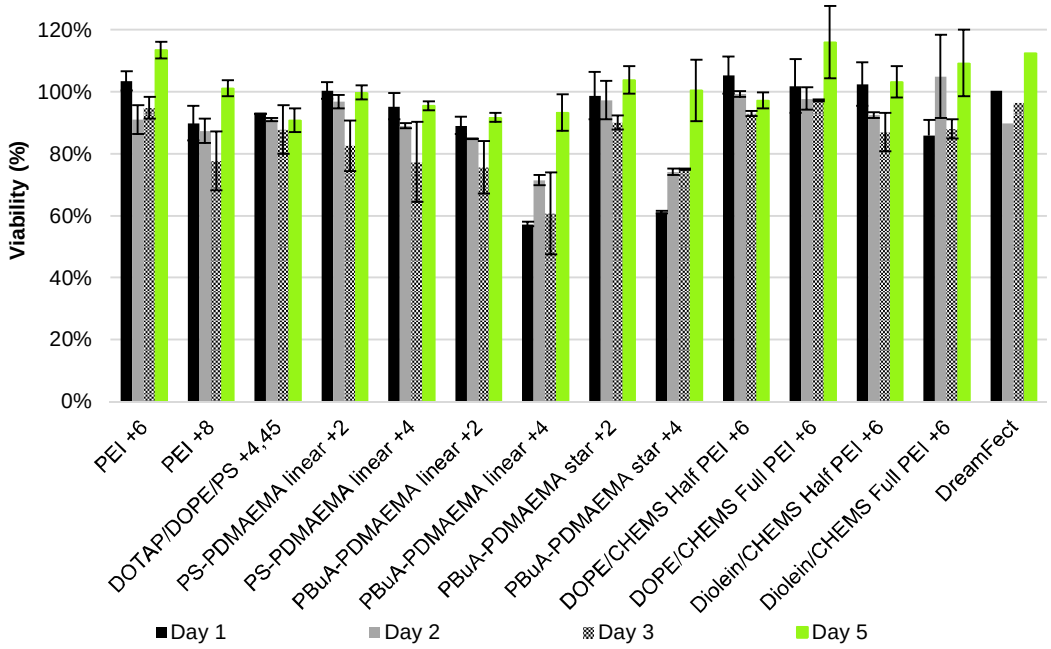

Figure 14. The viability of human primary RPE cells after transfection with carrier/plasmid complexes (±SEM). The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

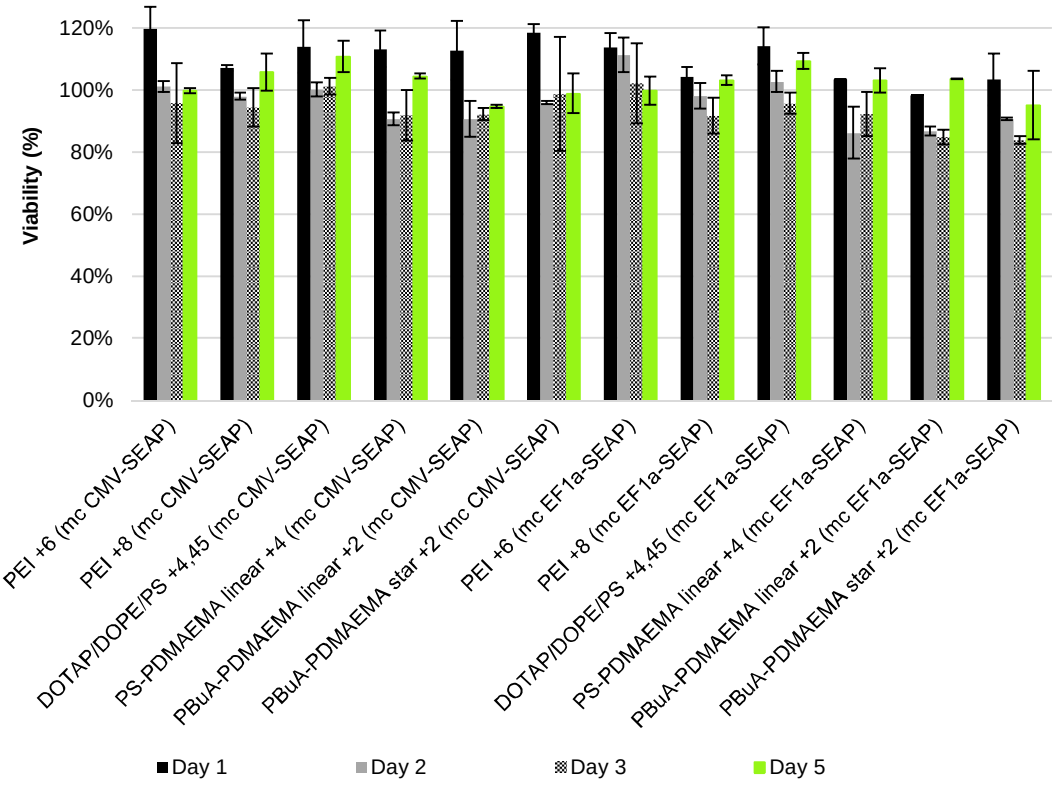

Figure 15. The viability of human primary RPE cells after transfection with carrier/minicircle complexes (±SEM). The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

11.4.4 Transfection of human embryonic stem cell-derived RPE cells

PEI/DNA complexes, copolymer/DNA polyplexes formed from linear PS-PDMAEMA, linear PBuA-PDMAEMA or star-like PBuA-PDMAEMA, and DOTAP/DOPE/PS/DNA lipoplexes were used in transfections complexed with plasmid DNA. The minicircles were used to form only PEI/DNA and DOTAP/DOPE/PS/DNA complexes. The cells were followed for 24 days after the first and 30 days after the second transfection (Figure 10). All the complexes turned out some transfection efficiency and the SEAP secretion rate of all the used complexes is shown in Figure 10.

The maximum SEAP secretion rate was almost at the same level between most of the transfections (0,1–1 ng/h/ml) (Figure 10). The highest SEAP secretion rate of ~18 ng/h/ml and the longest secretion period was reached with DOTAP/DOPE/PS liposomes complexed with plasmid. The cells transfected with DOTAP/DOPE/PS liposomes complexed with plasmid or EFa1-SEAP minicircle reached higher SEAP secretion level after the transfection compared with the other complexes. The duration of the SEAP secretion was also longer when the cells were treated with the concerned lipoplexes. The marker protein secretion started to wane significantly after 13 days after the transfections. Only a low SEAP signal from the cells treated with PEI/plasmid complexes at n/p ratio +6 could be detected on the day 30.

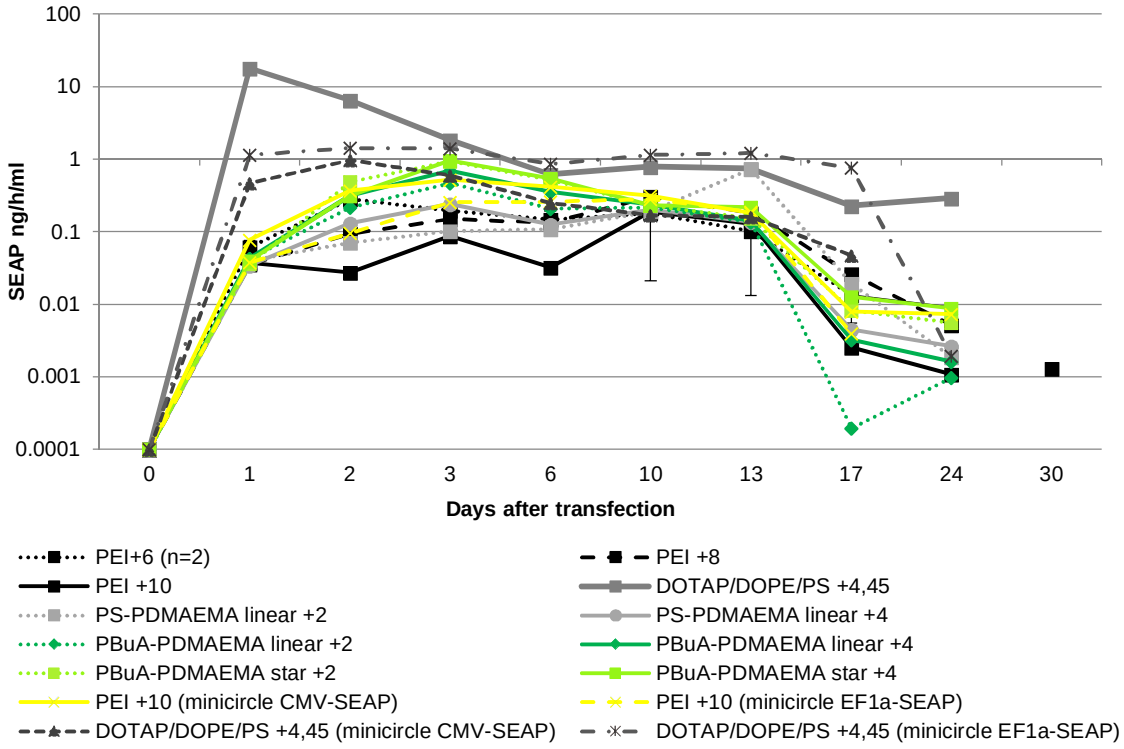

Figure 10. The SEAP secretion rate (ng/h/ml ±SEM) on the logarithmic scale after the transfection with carrier/DNA complexes to hESC-RPE cells. The results from two transfections are combined in the figure.

The metabolic activity of hESC-RPE was assessed with the AlamarBlue test (Figure 11). The viability of the cells did not decrease under 65 % after the transfections with any complexes. All the cells reached the viability of ~100 % after a few days from the transfection. However, copolymer/DNA complexes at high n/p ratios were not used for transfection of hESC-RPE and thus, the data from their impact on the cell viability is not available.

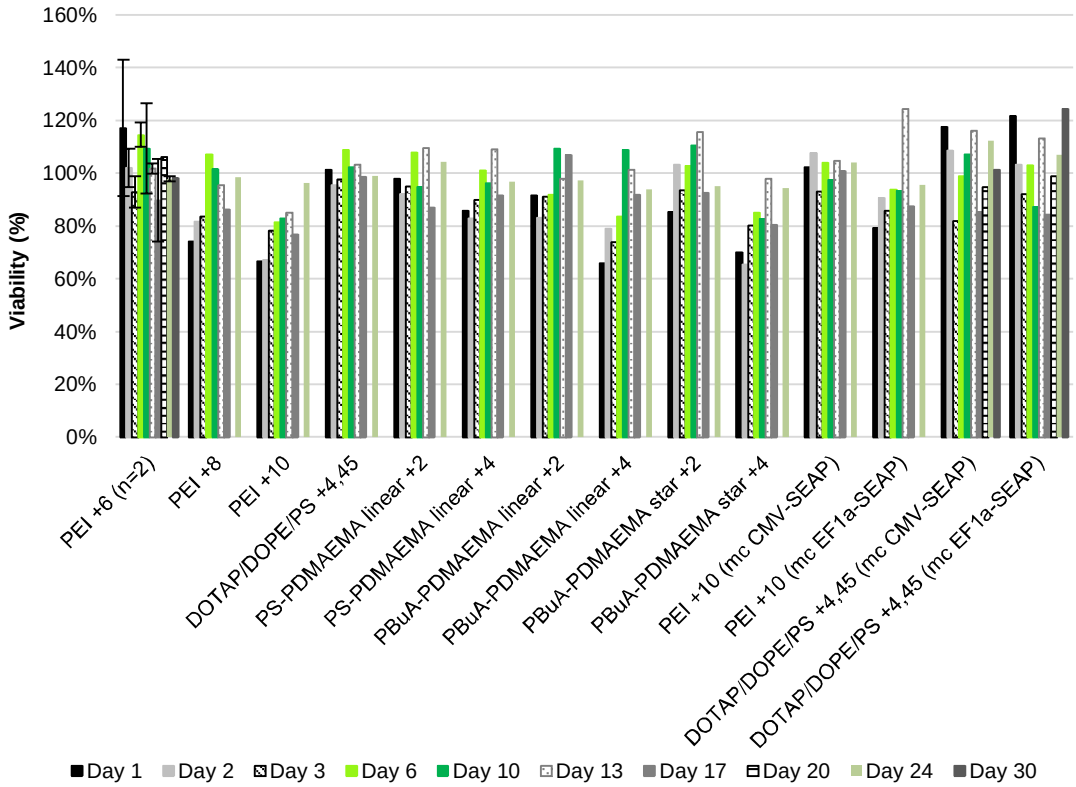

Figure 11. The viability of hESC-RPE after the transfections (±SEM). The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

11.4.5 Transfection of endothelial cells

Two transfections were conducted to EaHy cells. EaHy cells start to die after growing confluent on the wells, which leads to a limited follow-up period after a transfection. The viability of cells was assessed with the AlamarBlue test and with the microscope, and SEAP sample taking was stopped when cell density was decreasing after the reached confluence. After the first transfection, the cells were followed for seven days, while after the second transfection the cells died before they reached the fifth following day. The cells with passage numbers 19 and 27 were used for the first and the second transfection, respectively.

Both plasmid (EBNA-CMV-SEAP) and minicircles (CMV-SEAP and EF1a-SEAP) were used in transfections. Plasmid was complexed with PEI at n/p ratios +6, +8 and +10, DOTAP/DOPE/PS liposomes at n/p ratio +4,45, star-like PS-PDMAEMA and linear and star-like PBuA-PDMAEMA copolymers at n/p ratios 2 and 4. Plasmid was

also condensed with DreamFect. The following LCDCs complexed with plasmid were formed: DOPE/CHEMS half lipid PEI +6, full lipid PEI +6 and twice lipid PEI +8, diolein/CHEMS half lipid PEI +6 and full lipid PEI +6, and DPPE/CHEMS half and full lipid PEI +6. PEI polymer at n/p ratio +8 and DOTAP/DOPE/PS/DNA liposomes at n/p ratio +4,45 were used to complex both the minicircles.

When the SEAP secretion reached its maximum value after the transfections, it stayed almost at the same level until the cells started to die (Figure 16). Transfection with carrier/minicircle complexes reached higher SEAP secretion level than complexes formed with plasmid. The transfection was repeated with complexes which turned out to have transfection efficiency in the first transfection. The complexes formed from linear PS-PDMAEMA at n/p ratio +2, star-like and linear PBuA-PDMAEMA at n/p ratio +4 and full and twice lipid LCDCs did not reach any transfection efficiency except for DPPE/CHEMS full lipid complexes. In Figure 16 is presented the SEAP secretion rate after the transfections with the complexes that were used in the first or the first and the second transfections. As against, into Figure 17 is collected the SEAP secretion data from the cells treated with the complexes that were used only in the second transfection.

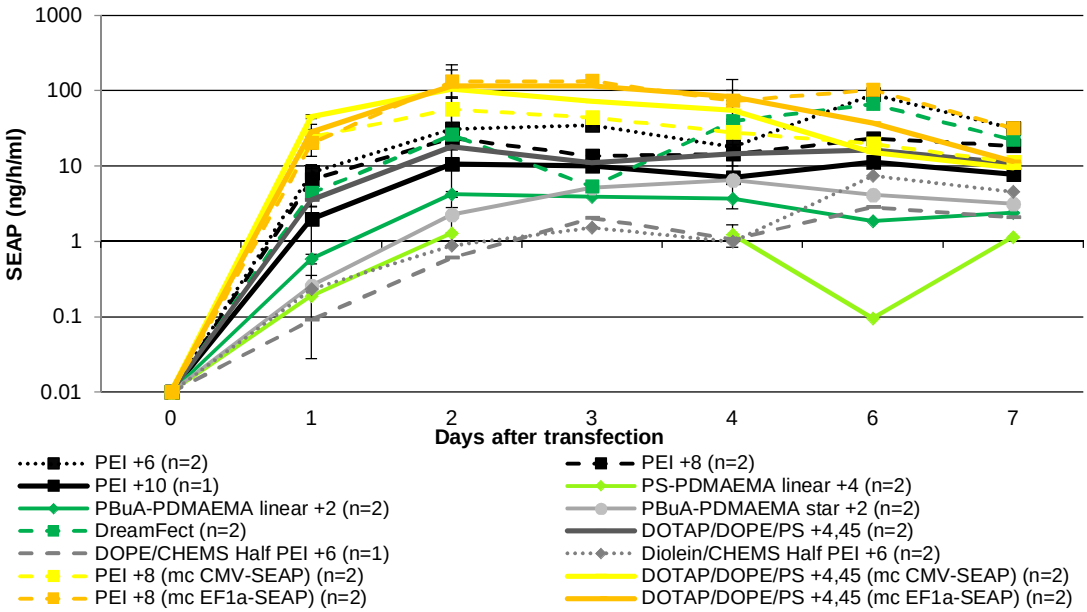

Figure 16. SEAP secretion (±SEM) on the logarithmic scale after the transfection to EaHy cells (Day 1–4: n=1 or 2, day 6–7 n=1). Both the plasmid and the minicircles (mc) were used in transfections. The data from the cells transfected with linear PS-PDMAEMA -based complexes is not shown on day 3 because of a mistake in sample taking.

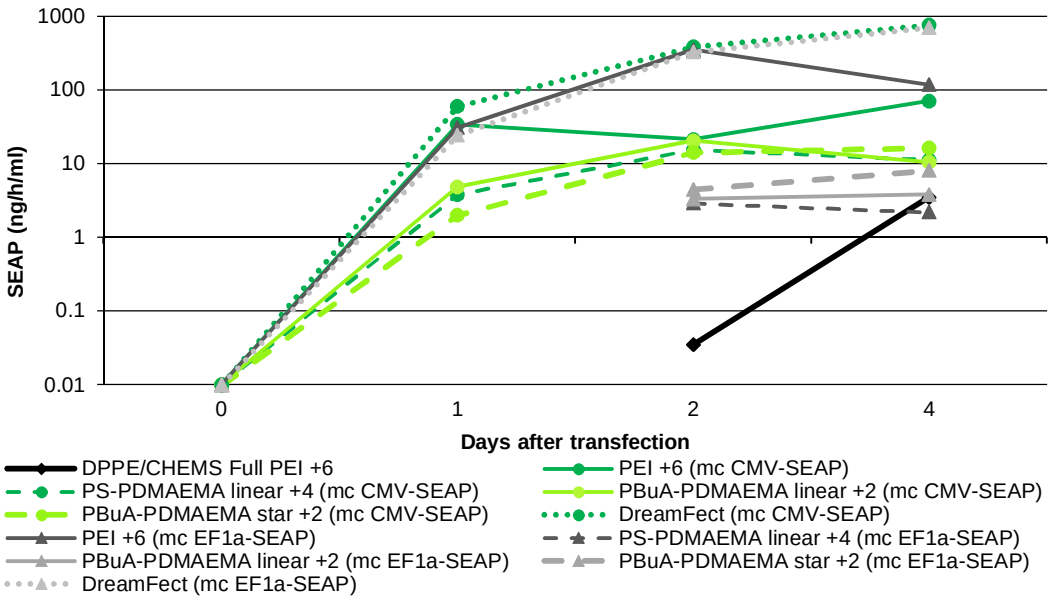

Figure 17. SEAP secretion after transfection with plasmid or minicircle to EaHy cells. CMV-SEAP or EF1a-SEAP minicircles (mc) were used to form complexes except to DPPE/CHEMS coated PEI/DNA complexes were formed with EBNA-CMV-SEAP plasmid.

The AlamarBlue test showed that the metabolic activity of EaHy cells after the first transfection decreased under the relative fluorescence value 50 % when the cells were transfected with many of the complexes (Figure 18). Generally, lipid-based complexes had higher viability compared with many polymer-based complexes. The cells recovered during the follow-up period except for the cells that were treated with star-like PBuA-PDMAEMA -based polyplexes at n/p +4 (Figure 18). After the second transfection, the cells reached the confluence earlier and the viability decreased earlier (Figure 19). Additionally, according to the microscope pictures the cell density was higher after the second transfection compared with the first one (Figure 7). The results from the AlamarBlue test after the first and second transfection are not combined because high differences in viability between the transfections were obtained.

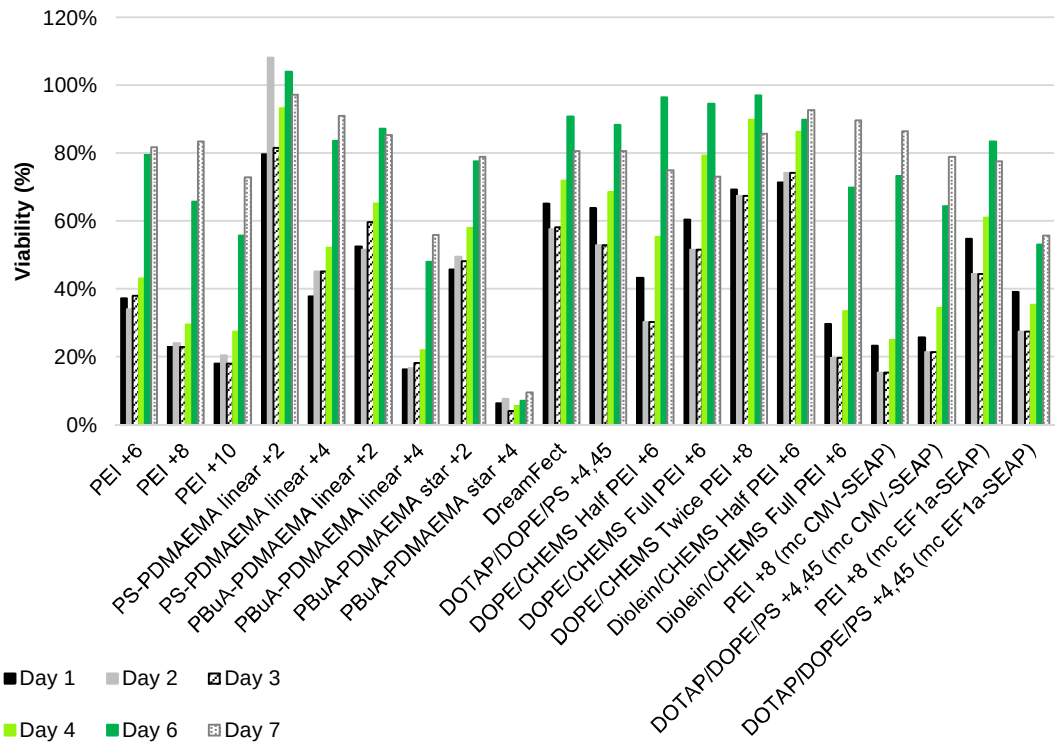

Figure 18. The viability of EaHy cells after the first transfection (p.19). The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

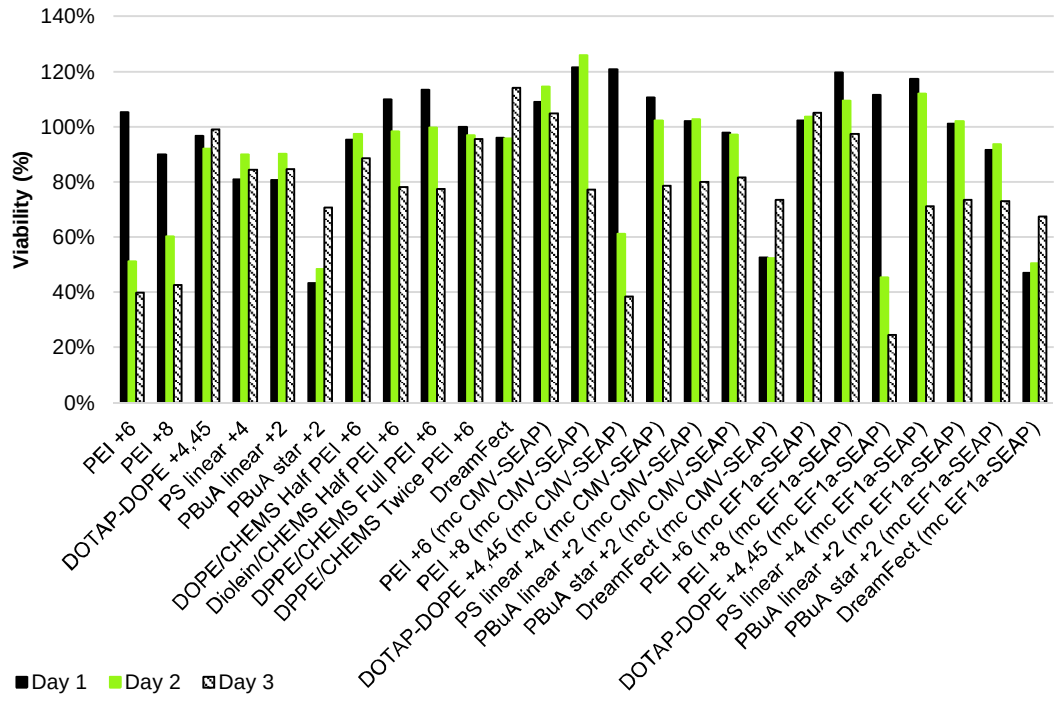

Figure 19. Viability of EaHy cells after the second transfection (p. 27). The metabolic activity is estimated with the AlamarBlue test. It is assumed that the transfection day is the day 0.

12 DISCUSSION

The eye is an optimal target for gene therapy because it is a well-defined, small organ and thus, with low drug doses is possible to obtain a therapeutic effect. Additionally, the eye is an immune-privileged site which means that the likelihood of immune responses provoked by gene delivery agents is decreased (Strauss 2005). In successful transfection, the carrier/DNA complexes need to fulfill the following requirements: 1) The complexes need to be stable enough; 2) The nanoparticles need to be taken up into the cells; 3) The carrier must protect DNA from the degradation. The balance between the complex sufficient stability and the minimal toxicity needs to be found to have efficient transfection. *In vivo* circumstances set more challenge compared with *in vitro* models because the complexes need to achieve the target tissue. In the case of AMD, the complexes are supposed to be administered intravitreally which means that they need to diffuse through the vitreous to reach the posterior eye.

12.1 Complex characterization

The carrier/DNA complexes are assumed to have interactions with the components of an anionic cell membrane like glycosaminoglycans (GAGs), which has impact on the cellular entry and stability of carrier/DNA-complexes (Ruponen et al. 2001). With a gel retardation assay and DNA relaxation assay, it can be studied how anionic components have impact on the complex stability. Anionic DS is assumed to mimic the influence of GAGs on the complexes and to have interactions with positively charged nanoparticles releasing DNA from the complexes. Size and zetapotential measurements give additional information on the complex formation and reproducibility of the process. The particle size has also been observed to have impact on the cell uptake mechanism and the transfection efficiency and thus, it is important to control it (Rejman et al. 2004; Masotti et al. 2009).

12.1.1 PEI/DNA polyplexes

In this study, PEI/DNA complexes condensed DNA efficiently when the n/p ratio was over +6 and DS broke the interactions between PEI and DNA efficiently

(Figure 2). The size of PEI/plasmid complexes at n/p ratio 6–10 was 161–180 nm and slightly bigger complexes were obtained when PEI was complexed with minicircles (Table 1). PEI complexes at n/p ratio +4 protected plasmid DNA in gel retardation assay but not in the DNA relaxation assay. In the DNA relaxation assay, complexes did not form, which can be seen in the high relative fluorescence value already before DS addition. According to the size measurement, the size of PEI +4 nanoparticles varied significantly between measurements and thus, the production of PEI/DNA complexes at n/p ratio +4 was not reproducible (Table 1). Due to the high deviation in the size of PEI/DNA complexes at n/p ratio +4, they were not selected for transfections to hESC-RPE, EaHy and human primary RPE cells. Controversially, in the other studies PEI has been shown to retard DNA, when the n/p ratio is higher than 1,5–2 (Honoré et al. 2005, Bertschinger et al. 2006). However, the calculation of n/p ratios might vary between studies and therefore the comparison of n/p ratios is difficult.

12.1.2 DOTAP/DOPE/PS/DNA lipoplexes

In the studies of Mannermaa et al. (2005), DOTAP/DOPE/PS/DNA lipoplexes were used at n/p ratio +4,45 in transfections to ARPE-19 cells. In addition to the n/p ratio +4,45, DOTAP/DOPE/PS/DNA lipoplexes with the higher n/p ratios were formed and characterized in this experimental work. However, in the gel retardation assay any difference between n/p ratios +4,45, +6 and +8 could not be detected (Figure 2). DS liberated DNA easily from the liposomes at any n/p ratio. The size of more positively charged DOTAP/DOPE/PS/DNA lipoplexes was ~70 nm smaller (130–136 nm) compared with the liposomes at n/p ratio +4,45 (203 nm) and the effect of higher n/p ratio and thus, the smaller size of nanoparticles was studied in the transfection to ARPE-19 cells.

12.1.3 PDMAEMA-based polyplexes

According to the gel retardation assay and the DNA relaxation assay, PBuA-PDMAEMA and PS-PDMAEMA micelles formed complexes with both plasmid and minicircle DNA (Figures 2, 3, 4, 5 and 6). Alhoranta et al. (2011) have earlier produced and studied the same copolymers, and they observed that both star-like and

linear PBuA-PDMAEMA and PS-PDMAEMA copolymers condense DNA in the gel retardation assay when the n/p ratio is 2 or higher. That is in the line with the results obtained with linear PBuA-PDMAEMA and PS-PDMAEMA -based polyplexes in this study.

Star-like PBuA-PDMAEMA micelles did not condense plasmid DNA efficiently and complexes were loose, which can be seen as bands on the agarose gel after the electrophoresis although DS is not present (Figure 2). The batch of star-like PBuA-PDMAEMA micelles might have impact on the condensation efficiency in this study: In the gel retardation assay of PBua-PDMAEMA/minicircle complexes, star-like PBuA-PDMAEMA compolymers condensed minicircles efficiently when a different micelle batch was used (Figure 4).

Linear PS-PDMAEMA and PBua-PDMAEMA copolymers protected plasmid DNA more strongly than star-like copolymers and DNA was released only partly from linear PDMAEMA-based complexes after the DS addition in electrophoresis (Figure 2). The same effect was observed in the studies of Alhoranta et al. (2011). The authors concluded that linear copolymers condense DNA more efficiently than star-like copolymers. Additionally, they observed the size of nanoparticles formed from linear copolymers was smaller in comparison with micelles produced from star-like copolymers. Also in this study, the Z-average size of linear copolymer/DNA polyplexes was smaller (120–245 nm) than star-like copolymer/DNA complexes (177–533 nm) except for the micelles formed with CMV-SEAP minicircle. Star-like and linear micelles complexed with CMV-SEAP minicircles had almost the same sizes (238–260 nm). The bigger size of polyplexes formed from star-like PDMAEMA-copolymers might result from the different geometry of polymers since star-like copolymers have six arms.

12.1.4 Lipid coated DNA complexes

Anionic LCDCs did not disintegrate after DS addition in the gel retardation assay and the DNA relaxation assay. As observed earlier, DOPE/CHEMS and diolein/CHEMS coated complexes protect DNA against DS but non-ionic surfactant Triton X-100 can liberate DNA from the complexes (Subrizi 2004; Lehtinen et al.

2008). The LCDCs are supposed not to disintegrate easily because a negatively charged lipid coat protects a positively charged PEI/DNA core (Lehtinen et al. 2008). However, with diolein/CHEMS and DOPE/CHEMS coated complexes faint bands can be seen at the end of the lanes as low as <0,5 kilobases in this study. The explanation for the presence of the bands is not known. Possibly, LCDCs released some DNA after the electrophoresis run.

The size of LCDCs was not as large as expected for the results of the size measurement of PEI/DNA polyplexes. Lehtinen et al. (2008) have observed that the size of polyplexes increased tens of nanometers after coating with DOPE/CHEMS lipid shield. In this study, the Z-average size of DOPE/CHEMS full lipid PEI +6 complexes was smaller than the Z-average size of PEI/DNA complexes at the n/p ratio +6 without coating (Table 1). The high polydispersity index of the Z-average value concerning LCDCs might explain this phenomenon. When there is a wide spectrum of particle size in the sample, Z-average value might not be an informative parameter to evaluate the particle size. The size of DPPE/CHEMS coated complexes was the highest varying between 430–800 nm.

12.2 Transfection efficiency of complexes

The secretion of marker protein, SEAP, was followed after transfections to RPE and endothelial cells to evaluate the efficiency of transfections. If the studied carrier/DNA complexes show sufficient potential to further studies, the marker gene can be replaced with a gene that expresses a therapeutic protein. For example, ranibizumab is an anti-angiogenic monoclonal VEGF-antibody that inhibits VEGF-A and it is used for the treatment of exudative AMD (Solomon et al. 2014). Thus, the gene that produces ranibizumab is a possible transgene for the gene delivery in exudative AMD. Ranibizumab has been observed to neutralize secreted VEGF at the minimum concentration of 60 ng/ml *in vitro* and this level gives some insight into a desirable level of a marker protein secretion to make the carrier/DNA complex potential for clinical applications (Klettner and Roider 2008). In this study encouraging results were received with many complexes after transfections concerning the possibility to further studies.

12.2.1 DOTAP/DOPE/PS/DNA lipoplexes

DOTAP/DOPE/PS lipoplexes reached or exceeded the transfection efficiency of the positive control in hESC-RPE, EaHy and human primary RPE cells and they showed low toxicicity in this study. Earlier DOTAP/DOPE/PS/DNA lipoplexes have been investigated in transfection studies in Chinese hamster ovary (CHO) cells and ARPE-19 cells also with encouraging results (Sorgi et al. 1997; Mannermaa et al. 2005). Condensing DNA with PS has been shown to increase the transfection efficiency of DOTAP/DOPE liposomes 12–222-fold compared DOTAP/DOPE liposomes alone in CHO cells (Sorgi et al. 1997). The condensation of DNA with PS has also shown to decrease cytotoxicity. The reason for that is supposed to lie in lower cationic lipid concentration when cationic PS neutralizes negatively charged DNA. Transfections with DOTAP/DOPE/PS/DNA lipoplexes to RPE cells was investigated by Mannermaa et al. (2005) and the secretion of SEAP in filter-cultured, differentated ARPE-19 cells was measured. According to the results, DOTAP/DOPE/PS/DNA lipoplexes (+4,45) had significant transfection efficiency compared with PEI/DNA complexes and the secretion of SEAP lasted almost two months.

In ARPE-19 cells, transfection with DOTAP/DOPE/PS/DNA lipoplexes was not successful but the SEAP secretion stayed at a low level in this study (Figure 8). Several n/p ratios of lipoplexes were examined in transfections to ARPE-19 cells but higher n/p ratios with smaller size did not transfect the cells. However, this was expected because the larger lipoplexes have been observed to have better transfection efficiency compared with the smaller ones (Masotti et al. 2009). The reason for the failed transfection with lipoplexes to ARPE-19 cells might lie in different transfection circumstances compared with the study of Mannermaa et al. (2005). Dividing ARPE-19 cells were used in this study as opposite to the study of Mannermaa et al. (2005) that was conducted with non-dividing, differentiated cells. Also the possibility of imperfect liposome formation before the transfection to ARPE-19 cells may be considered.

Despite unexpected results in ARPE-19 cells, in hESC-RPE, EaHy and human primary RPE cells were more successful. In EaHy cells DOTAP/DOPE/PS/minicircle lipoplexes had almost ten times higher transfection

efficiency (~115 ng/h/ml) on days 1–3 after transfection compared with DOTAP/DOPE/PS/plasmid lipoplexes (Figure 16). Contrary, in hESC-RPE and human primary RPE cells the liposomes complexed with plasmid had higher transfection efficiency (18 ng/h/ml and 12 ng/h/ml, respectively) and the secretion of a marker protein lasted longer compared with the transfection with DOTAP/DOPE/PS/minicircle lipoplexes (Figures 12, 13 and 16).

The marker protein secretion lasted about 20 days from the transfection day in hESC-RPE but the secretion of the marker protein stayed as low as under 20 ng/ml/h and even EF1a promoter could not prevent the silencing of the transgene expression. EF1a is a strong promoter of human origin and it is widely present in many human tissues, while CMV has virus origin (Uetsuki et al. 1989, Boshart et al. 1985). Based on their different backgrounds, EF1a is supposed to have longer transfection efficiency compared with CMV promoter in transfections to stem cells resulting from the silencing phenomenon of the transgene when a CMV promoter is used (Liu et al. 2009). However, the superiority of EF1a promoter over CMV promoter was not illustrated in this study.

12.2.2 PDMAEMA-based polyplexes

The transfection efficiency of PDMAEMA-based block copolymers was earlier evaluated in ARPE-19 and CV1-P cells (Alhoranta et al. 2011). When the transfection efficiency was studied with β-galactosidase gene, PBuA-PDMAEMA copolymers at n/p ratios +2 and +4 showed the highest expression level of the marker protein. In this study, PBuA-PDMAEMA-based polyplexes were also efficient in the transfections and thus, the results obtained in this study support the earlier observations but some differences are still found.

In ARPE-19 cells linear PBuA-PDMAEMA -based polyplexes at n/p ratio +2 reached the SEAP secretion level of ~450 ng/h/ml while star-like PBuA-PDMAEMA -based complexes showed to have only low ability to transfect ARPE-19 cells in this study. In the study of Alhoranta et al. (2011) controversially results were obtained concerning the architecture of the polymers: Star-like PBuA-PDMAEMA -based polyplexes did not show any transfection efficiency and successful results were

obtained with linear PBuA-PDMAEMA -based complexes in ARPE-19 cells. The reason for the low transfection efficiency of star-like PBuA-PDMAEMA -based complexes in ARPE-19 cells in this study might lie in the problems in micelle formation. If the micelles were not formed properly, the transfection efficiency remained low. New micelle solutions were made for the other transfections and higher transfection efficiency was observed with the other cell types.

The transfection to hESC-RPE and human primary RPE was successful with PDMAEMA-based polyplexes: both star-like and linear PBua-PDMAEMA -based and linear PS-PDMAEMA -based polyplexes at n/p ratio 2 and 4 reached the transfection efficiency of PEI/DNA polyplexes. Significant differences in the transfection efficiency or the duration of SEAP secretion between different polyplexes or n/p ratios were not observed in hESC-RPE. In human primary RPE cells, star-like PBuA-PDMAEMA -based complexes at n/p ratio +2 showed to have high transfection efficiency compared with the other complexes reaching the maximal transgene secretion rate of 50 ng/ml/h. The SEAP was also secreted longer when human primary RPE cells were transfected with PDMAEMA-based polyplexes compared with PEI/DNA complexes. The cells were followed only for 18 days and the secretion of SEAP was still detectable when the sample taking was stopped. Thus, it was not got an answer to the question how long the cells could have secreted SEAP after the transfection with PDMAEMA-based carriers in human primary RPE cells in this study.

CMV-SEAP minicircle showed better transfection efficiency in human primary RPE and EaHy cells compared with polyplexes with plasmid. The transfection efficiency of PBuA-PDMAEMA-based polyplexes at n/p ratio +2 with CMV-SEAP minicircle was on days 1–3 from the transfection about two times higher than the transfection efficiency of corresponding complexes with plasmid in human primary RPE cells. In EaHy cells, linear and star-like PBuA-PDMAEMA -based polyplexes at n/p ratio +2 and linear PS-PDMAEMA -based polyplexes at n/p ratio +4 with CMV-SEAP minicircle showed ~3,5–10 times higher transfection efficiency compared with corresponding plasmid complexes depending on the carrier.

Cytotoxicity has been observed as a problem with the PDMAEMA-based carriers at least at higher n/p ratios and it has led to the modifications of PDMAEMA polymers (van de Wetering et al. 1998, Alhoranta et al. 2011). The n/p ratio needs to be high enough for proper complex formation but not rise too high because of high cytotoxicity (van de Wetering et al. 1998). In EaHy cells, micelles reached only low transfection efficiency and the reason why PDMAEMA-based complexes did not achieve better results might lie in high cytotoxicity. The viability of EaHy cells decreased strongly after transfection with PDMAEMA-based complexes. Additionally, the viability of ARPE-19 cells was studied when the cells were transfected with PDMAEMA-based polyplexes at the wide range of n/p ratios from +2 to +16. It was shown that high n/p ratios +8 and +16 had harmful effects on the metabolic activity of the cells. When n/p ratio +16 was used, not any transfection efficiency was observed in ARPE-19 cells and the reason for that is probably the high cytotoxicity.

12.2.3 Lipid coated DNA complexes

The transfection efficiency of LCDCs was evaluated in EaHy and human primary RPE cells in this study. In EaHy cells, DOPE/CHEMS, diolein/CHEMS and DPPE/CHEMS coated complexes did not reach the transfection efficiency of uncoated PEI and even ~10–30 times lower transfection efficiency was observed in comparison with uncoated PEI +6 complexes. In human primary RPE cells LCDCs did not reach any detectable transfection efficiency.

Subrizi (2004) has studied the transfection efficiency of diolein-CHEMS coated LCDCs in ARPE-19 cells and LCDCs did not show any transfection efficiency in that study. However, the transfection with diolein/CHEMS full lipid PEI/DNA +8 was successful to monkey kidney fibroblast (CV1-P) cells and 2,5 times higher transfection efficiency compared with uncoated PEI/DNA +8 complexes was detected. DOPE/CHEMS coated complexes was also studied in CV1-P cells but efficient transfection was not observed (Lehtinen et al. 2008). Lehtinen et al. (2008) also showed that PEGylation of LCDCs increased the cell uptake, but transfection efficiency was lower with PEGylated complexes compared with LCDCs without coating. However, studies in KB cell line, which is a subline of the ubiquitous

keratin-forming tumor cell line HeLa, showed that DOPE/CHEMS and DOTAP/CHEMS coated PEI/DNA complexes at n/p ratio 4 have higher transfection efficiency compared with uncoated polyplexes (Guo et al. 2002). The reason for the controversial results might lie in the differences in the cell uptake of LCDCs between the cell lines. If LCDCs can not enter the RPE cells, it can probably explain why LCDC are not efficient gene delivery vehicles in RPE cells.

In this study, LCDCs with a new lipid composition of LCDC was also examined when DPPE lipid was mixed with CHEMS to form the lipid shield to PEI/DNA complexes. It was earlier shown that DPPE might reach a hexagonal state which is a desired property concerning endosomal escape of the complexes in the cell (Ramezani et al. 2009). However, the transfection efficiency of the complexes with DPPE/CHEMS lipid shield stayed at low level in EaHy cells. One reason for that might be the large Z-average size of nanoparticles that was 433–800 nm depending on the complex.

12.3 Future perspectives

The further development of PBuA-PDMAEMA-based complexes with episomal plasmid or minicircle with CMV-promoter for transfections to RPE cells is justified according to this study because positive results were obtained compared with positive control and reasonable marker protein secretion levels were obtained. However, the possible toxicity of PDMAEMA-based polymers needs to be taken account. Because human primary RPE cells and ARPE-19 cells were followed only for 18 and 6 days, respectively, more information is needed to evaluate the duration of the marker protein secretion before their potential to *in vivo* studied can be confirmed. In addition, all the experiments were conducted with dividing RPE cells in this study. The transfection of non-dividing cells is more challenging and RPE cells do not divide *in vivo*, which leads to a need to investigate the transfection efficiency of carrier/DNA complexes also in non-dividing RPE cells.

The attractive results were obtained when EaHy cells and hESC-RPE were transfected with DOTAP/DOPE/PS/DNA lipoplexes with episomal plasmid and minicircle with EF1a-promoter. However, in hESC-RPE transfection efficiciency

lasted only 20 days and the secretion of marker protein stayed at low level. Concerning the cell replacement therapy, the possibility to transfect hESC-RPE cells before their transplantation to produce therapeutic protein would not be efficient according to the results with the carriers studied in this experimental work. However, the further transfection studies to endothelial cells with DOTAP/DOPE/PS/DNA lipoplexes would be interesting and the transfection of endothelial cell line that could be followed longer time could give more information on the transgene expression duration.

13 CONCLUSIONS

The focus of this study was to investigate gene delivery to the diseases of the posterior eye. Non-viral transfection to RPE cells and endothelial cells was studied *in vitro* using several combinations of carriers and DNA forms. According to the results, it can be concluded that PBuA-PDMAEMA-based complexes showed to have high transfection efficiency in human embryonic primary RPE cells and ARPE-19 cells, while attractive results were obtained when EaHy cells were transfected with DOTAP/DOPE/PS/DNA lipoplexes. In hESC-RPE, DOTAP/DOPE/PS/DNA complexes exceeded the transfection efficiency of the positive control but the marker protein secretion level remained low lasting ~20 days. Transefctions did not succeed with LCDCs. In addition to episomal plasmid, minicircles showed to be interesting DNA forms for transfections and in hESC-RPE and EaHy cells the high transfection efficiency was achieved when the minicircle with EF1a promoter was used in transfections, while minicircle with CMV promoter showed to be more efficient in transfections to human primary RPE cells. However, more investigation is needed to obtain more information on the duration of transgene expression in RPE and endothelial cells and the transfection efficiency of the complexes in non-dividing cells.

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